PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 91357	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International application No. **PCT/AU 98/00930**	International filing date *(day/month/year)* 6 November 1998	Priority Date *(day/month/year)* 6 November 1997
International Patent Classification (IPC) or national classification and IPC **Int. Cl.**[6] C12N 15/63		
Applicant COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand 19 April 1999	Date of completion of the report 3 August 1999
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (02) 6285 3929	Authorized Officer **PHILIPPA WYRDEMAN** Telephone No. (02) 6283 2554

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/AU 98/00930

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , filed with the letter of .

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages

[ ] the claims, Nos.

[ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	**1-19**	**YES**
	Claims	**None**	**NO**
Inventive step (IS)	Claims	**1-19**	**YES**
	Claims	**None**	**NO**
Industrial applicability (IA)	Claims	**1-19**	**YES**
	Claims	**None**	**NO**

2. Citations and explanations (Rule 70.7)

The invention as defined by claims 1-19 resides in the combination of a heterologous peptide linked to a first promoter, a restriction enzyme linked to an inducible promoter, and a cleavage site for said restriction enzyme which is absent from the chromosomal DNA of the host cell. The heterologous peptide, when introduced into a host cell, will be expressed until the expression of the restriction enzyme is induced and the cleavage of the suicide expression vector is brought about.

All of citations are directed towards killing the host cell when the suicide gene is expressed rather than destroying only the vector per se. Therefore, the invention defined by claims 1-19 is taken to be novel, involve and inventive step over the prior art and have industrial applicability.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/63

A1

(11) International Publication Number: WO 99/24590

(43) International Publication Date: 20 May 1999 (20.05.99)

(21) International Application Number: PCT/AU98/00930

(22) International Filing Date: 6 November 1998 (06.11.98)

(30) Priority Data:
PP 0215 6 November 1997 (06.11.97) AU

(71) Applicants *(for all designated States except US)*: COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). THE AUSTRALIAN NATIONAL UNIVERSITY [AU/AU]; Canberra, ACT 2601 (AU). AGRICULTURE PROTECTION BOARD OF WESTERN AUSTRALIA [AU/AU]; Baron–Hay Court, South Perth, W.A. 6151 (AU). DEPARTMENT OF CONSERVATION AND LAND MANAGEMENT [AU/AU]; Hackett Drive, Crawley, W.A. 6009 (AU).

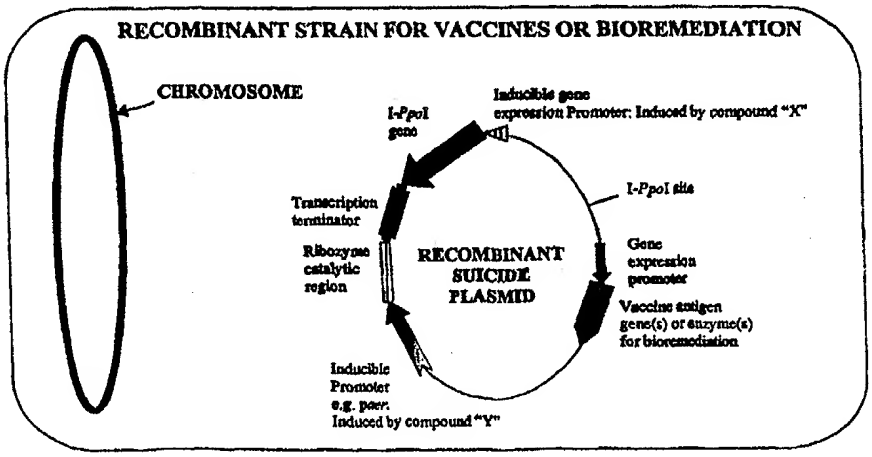
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BRAHMBHATT, Himanshu, N. [IN/AU]; 96 Prairie Vale Road, Bossley Park, NSW 2176 (AU). SEYMOUR, Robert, B. [AU/AU]; 1/14 Newline Road, West Pennant Hills, NSW 2125 (AU). BIRD, Peter, H. [AU/AU]; Unit 4, 52 Ormond Street, Turner, ACT 2601 (AU). BRADLEY, Mark, P. [AU/AU]; 59 Calley Drive, Leeming, W.A. 6149 (AU).

(74) Agent: F.B. RICE & CO.; 605 Darling Street, Balmain, NSW 2041 (AU).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: SUICIDE EXPRESSION VECTOR FOR VACCINE STRAINS



(57) Abstract

A suicide expression vector for expressing a heterologous peptide, polypeptide or protein in a selected host cell, said vector comprising: (i) a first nucleotide sequence encoding said heterologous peptide, polypeptide or protein (e.g. sperm antigens, esterases capable of hydrolysing organophosphates and insecticidal toxins) operably linked to a first promoter sequence; (ii) a second nucleotide sequence encoding a restriction enzyme or functional portion thereof operably linked to a second promoter sequence, said second promoter sequence being inducible; and (iii) one or more cleavage site(s) for said restriction enzyme or functional portion thereof, said cleavage site(s) being absent from the chromosomal DNA of said host cell, wherein upon introduction of the vector into said host cell, induced expression of the restriction enzyme or functional portion thereof from said second nucleotide sequence brings about the cleavage of the suicide expression vector. The vector is useful for the production of microorganisms intended for environmental release in the applications of, for example, vaccine baits for sterilisation of vermin, compositions for bioremediation of contaminated soils and waste, and insecticidal compositions for crop spraying.

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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00930

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/63 - 15/86 + SUICID: OR DEATH OR KILL:

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: Keywords as above
Chemabs: Plasmid and episome/ct

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A, 9534643 (WACKERNAGEL W) 21 December 1995	
A	Appl. Environ. Microbiol., v 60, no. 10, Oct 1994, pp 3746-51, Ahrenholtz I *et al*, "A conditional suicide system in Escherichia coli based on the intracellular degradation of DNA"	
A	Curr. Opin. Biotechnol., v 4, no. 3, Jun 1993, pp 299-305, Molin S, "Environmental potential of suicide genes"	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1998	16 DEC 1998
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **BARRY SPENCER** Telephone No.: (06) 283 2284

International Application No.
PCT/AU 98/00930

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Gene, v 118, no. 1, 1992, pp 145-6, Penfold R *et al*, "An improved suicide vector for construction of chromosomal insertion mutations in bacteria"	
A	Gene, v 29, no. 1-2, 1984, pp 63-8, Kovacs B *et al*, "The generation of a single nick per plasmid molecule using restriction endonucleases with multiple recognition sites"	

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00930

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	9534643	EP	717775

END OF ANNEX

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Published
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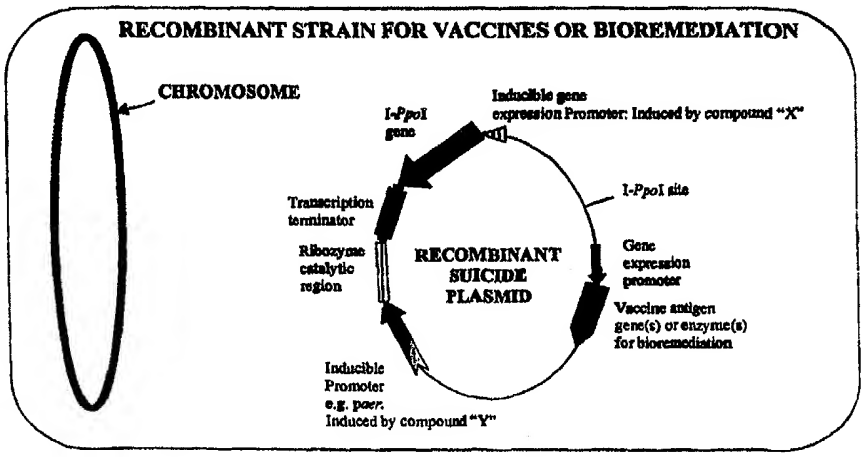
(54) Title: SUICIDE EXPRESSION VECTOR FOR VACCINE STRAINS



(57) Abstract

A suicide expression vector for expressing a heterologous peptide, polypeptide or protein in a selected host cell, said vector comprising: (i) a first nucleotide sequence encoding said heterologous peptide, polypeptide or protein (e.g. sperm antigens, esterases capable of hydrolysing organophosphates and insecticidal toxins) operably linked to a first promoter sequence; (ii) a second nucleotide sequence encoding a restriction enzyme or functional portion thereof operably linked to a second promoter sequence, said second promoter sequence being inducible; and (iii) one or more cleavage site(s) for said restriction enzyme or functional portion thereof, said cleavage site(s) being absent from the chromosomal DNA of said host cell, wherein upon introduction of the vector into said host cell, induced expression of the restriction enzyme or functional portion thereof from said second nucleotide sequence brings about the cleavage of the suicide expression vector. The vector is useful for the production of microorganisms intended for environmental release in the applications of, for example, vaccine baits for sterilisation of vermin, compositions for bioremediation of contaminated soils and waste, and insecticidal compositions for crop spraying.

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SUICIDE EXPRESSION VECTOR FOR VACCINE STRAINS

Field of the Invention:

The present invention relates to suicide expression vectors particularly for use in the production of microorganism vectors intended for environmental release. In a particular application of the invention, the expression vector is used in the production of a bacterial vaccine bait for sterilisation of vermin species.

Background of the Invention:

There is a worldwide concern regarding the environmental release of genetically engineered microorganisms (GEMs) (Wilson & Lindow, 1993). Microorganisms harbour efficient mechanisms for horizontal gene transfer that enable them to adapt to environmental changes. Conjugation, transduction, transformation and retromobilization are the main mechanisms that contribute to the flux of genes within microbial communities (Veal *et al.*, 1992).

In recent times, conditional suicide systems have been developed to address this problem, particularly for use in GEMs involved in bioremediation of contaminated soils and in waste treatment. These systems however, involve the use of inducible lytic systems which lyse the bacterial cell (e.g. Bej *et. al.*, 1988). Such systems would not be applicable to live recombinant vaccines that use bacterial delivery vectors since they would lyse the bacterial cells prior to vaccination. For such vaccines to be successful in eliciting antigen-specific immune responses, they must be live. Early work has demonstrated that killed bacterial vectors are not as effective as live ones. Such recombinant vaccines if used in baits could pose the hazard of transferring the recombinant DNA to other organisms in the environment.

In order to overcome this problem, the present inventors have developed a suicide expression vector system which results in the selective degradation of the recombinant expression vector DNA without destroying chromosomal DNA, prior to release of the microorganism vector into the environment.

Disclosure of the invention:

Thus, in a first aspect, the present invention provides a suicide expression vector for expressing a heterologous peptide, polypeptide or protein in a selected host cell, said vector comprising:

(i) a first nucleotide sequence encoding said heterologous peptide, polypeptide or protein operably linked to a first promoter sequence,

(ii) a second nucleotide sequence encoding a restriction enzyme or functional portion thereof operably linked to a second promoter sequence, said second promoter sequence being inducible, and

(iii) one or more cleavage site(s) for said restriction enzyme or functional portion thereof, said cleavage site(s) being absent from the chromosomal DNA of said host cell,

wherein upon introduction of the vector into said host cell, induced expression of the restriction enzyme or functional portion thereof from said second nucleotide sequence brings about the cleavage of the suicide expression vector.

The suicide expression vector according to the present invention may be transformed into a host cell, preferably a bacterial or yeast host cell, and used to produce a desired heterologous peptide, polypeptide or protein. Once sufficient expression of the heterologous peptide, polypeptide or protein has occurred, the transformed host cell may be induced to express the restriction enzyme or functional portion thereof thereby causing the cleavage and subsequent degradation of the expression vector.

The host cell and the restriction enzyme/cleavage site(s) are selected so as to ensure that the expression of the restriction enzyme brings about the cleavage of the recombinant expression vector only. In other words, the host cell and restriction enzyme/cleavage site(s) are selected so as to ensure that the host's DNA is not cleaved by the expressed restriction enzyme. Selection may be readily made by isolating host DNA from a test microorganism by known methods, subjecting the isolated host DNA to a candidate restriction enzyme under suitable conditions, and analysing the host DNA by, for example, gel electrophoresis for any cleavage. If no cleavage has occurred then this should indicate that the host DNA does not include a cleavage site for the candidate restriction enzyme. Preferably, the restriction enzyme is selected from those that recognise cleavage sites of ten or more nucleotides such as I-*Ppo*I, I-*Ceu*I, P1-*Psp*I, P1-*T1i*I and P1-*Sce*I.

The first promoter sequence may be a constitutive promoter sequence but, more preferably, is an inducible promoter. However, the second promoter sequence must, as stated above, be an inducible promoter. Moreover, it is an important feature of the present invention that the second promoter sequence is not excessively "leaky" prior to induction, since premature expression of the restriction enzyme or functional portion thereof may lead to the cleavage and subsequent degradation of the expression vector.

Induction of the second promoter sequence may be achieved by providing an inducer molecule that interacts with a protein which represses transcription. Examples of a promoter able to be induced in this manner are the p*lacZ* promoter, the p*lacUV5* promoter and the T7 RNA polymerase promoter. Other methods for inducing the inducible second promoter sequence may be through exposure to UV radiation or heat shock or environmental stress such as modulation of concentration of nutrients, oxygen, pH etc. However, preferably, the inducible second promoter sequence is induced through the expression of a peptide, polypeptide or protein required to initiate or otherwise cause transcription from the second promoter sequence. In this manner of induction, expression of the peptide, polypeptide or protein required for transcription, is placed under the control of an inducible promoter such as those mentioned above.

Thus, in preferred embodiments of the invention, the second promoter sequence is a promoter sequence which is unrecognised by the RNA polymerase(s) of the host cell, and the expression vector further comprises an additional nucleotide sequence encoding an RNA polymerase for the second promoter sequence operably linked to an inducible promoter.

In a particularly preferred embodiment of the invention, the second promoter sequence is a T7 RNA polymerase promoter sequence which is unrecognised by the RNA polymerase of the host cell and the expression vector further comprises a nucleotide sequence encoding T7 RNA polymerase operably linked to an inducible promoter sequence such as the *lacUV5* promoter which is induced by isopropyl-β-thiogalactopyranoside (IPTG) (alternatively, the host cell includes on its chromosome(s) or on a plasmid(s) a nucleotide sequence encoding T7 RNA polymerase operably linked to an inducible promoter sequence such as the p*lacUV5* promoter). However, since the p*lac*UV5 promoter is prone to "leakage", the expression

vector of this preferred embodiment may also comprise a nucleotide sequence encoding lysozyme expressibly linked to a constitutive promoter sequence. Constitutive expression of the lysozyme will inhibit the "leaky" expression of T7 RNA polymerase and thereby prevents the premature expression of the restriction enzyme or functional portion thereof.

As a "safeguard" against premature expression of the restriction enzyme or functional portion thereof, the expression vector preferably further comprises a nucleotide sequence encoding a ribozyme targetted against the mRNA produced from the nucleotide sequence encoding the restriction enzyme or functional portion thereof. The ribozyme encoding nucleotide sequence is operably linked to a constitutive or inducible promoter sequence. The ribozyme should be expressed such that it will be present to immediately cleave low "leakage" amounts of mRNA encoding the restriction enzyme or functional portion thereof. Induced expression of the restriction enzyme or functional portion thereof will overwhelm the cleavage activity of the ribozyme and thus result in the cleavage and subsequent degradation of the expression vector.

In a second aspect, the present invention provides a host cell transformed with a suicide expression vector according to the first aspect.

In a third aspect, the present invention provides a method of expressing a heterologous peptide, polypeptide or protein in a selected host cell, comprising;

(i) transforming said host cell with a suicide expression vector according to the first aspect,

(ii) culturing said transformed host cell under suitable conditions for the expression of the said heterologous peptide, polypeptide or protein, and

(iii) thereafter inducing expression of the restriction enzyme or functional portion thereof to bring about cleavage of the said suicide expression vector.

In a fourth aspect. the present invention provides a method for the production of a microorganism vector which contains recombinant peptide, polypeptide or protein but no recombinant DNA, comprising:
(i) transforming said microorganism with a suicide expression vector according to the first aspect,
(ii) culturing said transformed microorganism under suitable conditions for the expression of the said heterologous peptide, polypeptide or protein, and
(iii) thereafter inducing expression of the restriction enzyme or functional portion thereof to bring about cleavage of the said suicide expression vector.

Preferably, the microorganism vector is intended for enviromental release in the applications of, for example, vaccine baits for sterilisation of vermin, vaccination against animal pathogens, compositions for bioremediation of contaminated soils and waste, and insecticidal compositions for crop spraying. In such applications, the heterologous peptide, polypeptide or protein contained in, or expressed on the surface of the microorganism may be, respectively, a zona pellucida or sperm or hormone antigen, bacterial, viral or parasite antigen, an esterase capable of hydrolysing organophosphates, and an insecticidal toxin such as Bt toxin.

In a fifth aspect, the present invention provides a microorganism vector produced by the method according to the fourth aspect.

The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated step, component or feature or group of steps, components or features with or without the inclusion of a further step, component or feature or group of steps, components or features.

The invention will hereinafter be described by way of the following non-limiting examples and accompanying figures.

Brief description of the accompanying figures:

FIGURE 1. PLASMID CONSTRUCTION.

The genetic construction of the various plasmids used in this study is shown. Only the relevant segments of each plasmid are shown. The oligonucleotides (RBS numbers) used for each construct are shown in italics. Plasmid names are shown in a box. The abbreviations used include: MCS, multiple cloning site; p*aer*, iron regulated aerobactin promoter; AmpR,

ampicillin resistance gene; p*T7*, phage T7 promoter: p*T3*, phage T3 promoter; p*Omp*A, *E. coli* outer membrane A promoter: p*lac*, *E. coli* lactose operon promoter: *lac*O, *E. coli* lactose operon operator: *lac*I, *E. coli* lactose operon repressor.

FIGURE 2. DEMONSTRATION OF FUNCTION OF I-*Ppo*I IN *E. coli*.

The plasmids pRBS 22 and pI3-941 were transformed into JM 109 (DE3) and examined for self-restriction upon induction with IPTG. Two isolates of pI3-941 and one of pRBS22 in JM109 (DE3) were grown to OD_{600} = 0.5, induced with 1mM IPTG for 1 hour and plasmid prepared by the alkaline lysis method. Plasmid profiles of uninduced and induced cultures are shown. The recombinant plasmid is indicated with an arrow. The molecular weight marker used is λ *Hind*III.

FIGURE 3. SYNTHETIC RIBOZYME CONSTRUCT.

The oligonucleotides RBS 67a, 68, 69, 70, 71, 72 were used to construct the ribozyme genetic cassette. Ribozyme expression is under the control of *omp*A promoter and transcription is terminated at the T7 terminator. The ribozyme targets bases 345-369 of the I-*Ppo*I intron 3 sequence (Muscarella et al., 1990). Restriction sites and other relevant regions are shown below the DNA sequence.

FIGURE 4. I-*Ppo*I ACTIVITY CONTROLLED WITH RIBOZYME.

Four different isolates of pRBS 43 were transformed into JM109 (DE3) and examined for plasmid maintenance in the uninduced state and for self-restriction of plasmid upon induction. The cells were grown to OD_{600} = 0.5 and induced with 1mM IPTG for 1 hour before plasmid was isolated from the cells. Plasmid profiles from uninduced and induced cells are shown. The molecular weight marker used is λ *Hind* III and the plasmid band is indicated with an arrow.

FIGURE 5. EFFECT OF GROWTH TEMPERATURE ON PLASMID SELF-RESTRICTION.

Three independent isolates of recombinant JM109 (DE3) strain carrying plasmid pRBS 45 were grown to OD_{600} = 0.5 either at 37°C or 42°C and all cultures were induced at 37°C with 1mM IPTG for 1 hour. Plasmid profiles

for each culture are shown. The plasmid is indicated with an arrow. λ *Hind* III molecular weight marker is also shown.

FIGURE 6. EFFECT OF THE IRON-REGULATED PROMOTER (*paer*) ON PLASMID SELF-RESTRICTION.

Six different isolates of pRBS 46 were transformed into JM109 (DE3) and examined for self restriction of plasmid upon induction. The cells were grown to OD_{600} = 0.5 and induced with 1mM IPTG for 1 hour before plasmid was isolated from the cells. Plasmid profiles of uninduced and induced cultures are shown. The molecular weight marker is λ *Hind* III and the plasmid band is indicated with an arrow.

FIGURE 7. EFFECT OF THE IRON-REGULATED PROMOTER (WITHOUT UP-STREAM PROMOTERS) ON PLASMID SELF-RESTRICTION.

Six different isolates of pRBS 47 were transformed into JM109 (DE3) and examined for self-restriction of plasmid upon induction. The cells were grown to OD_{600} = 0.5 and induced with 1mM IPTG for 1 hour before plasmid was isolated from the cells. Plasmid profiles from uninduced and induced cells are shown. The molecular weight marker λ *Hind* III is shown and the plasmid band is indicated with an arrow.

FIGURE 8. DEMONSTRATION OF EFFECTIVE SUICIDE PLASMID.

Six different isolates of pRBS 48 were transformed into JM109 (DE3) and examined for self-restriction of plasmid upon induction. The cells were grown to OD_{600} = 0.5 and induced with 1mM IPTG for 1 hour before plasmid was isolated from the cells. The molecular weight marker λ *Hind* III is shown and the plasmid band is indicated with an arrow.

FIGURES 9A TO 9F. OVERVIEW OF THE BASIC CONCEPT OF THE SUICIDE PLASMID FOR VACCINE AND/OR BIOREMEDIATION STRAINS.

The complete concept is outlined in the figures 9A to 9F which show the sequential steps involved in initially expressing large quantities of the recombinant protein followed by elimination of all recombinant DNA.

TABLE 1. LIST OF OLIGONUCLEOTIDES.

The DNA sequence and relevant details for each of the synthetic oligonucleotides used in this study are shown.

OLIGONUCLEOTIDE	DNA SEQUENCE
RBS 3 (SEQ ID NO. 1)	5' Spacer: AGCT SmaI: CCCGGG I-PpoI from 5' end: CTCTCTTAAGGTAG SmaI: CCCGGG Spacer: AGCT 3'
RBS 4 (SEQ ID NO. 2)	5' Spacer: AGCT SmaI: CCCGGG I-PpoI from 3' end: CTACCTTAAGAGAG SmaI: CCCGGG Spacer: AGCT 3'
RBS 67a (SEQ ID NO. 3)	5' Spacer: GAATC XhoI: CTCGAG OmpA promoter start: GAGTTCACATTGTAAGTTTTC 3'
RBS 68 (SEQ ID NO. 4)	5' Spacer: AAGAG BglII: AGATCT OmpA promoter from 3' end: AGTCTACAACGTAGTTGAAAACTTACAATGTGAACTCC 3'
RBS 69 (SEQ ID NO. 5)	5' Spacer: AGACT BglII: AGAT I-PpoI from 5' end: CTCTCTTAAGGTAGC Region 369-358 of I-PpoI gene: TTGTCGTCTAGT Catalytic domain of Ribozyme from 5' end: CTGATGAGTCCG 3'
RBS 70 (SEQ ID NO. 6)	5' AGGGGTTATGTGTGCTGGGAGTTTCGTCCTCACGGACTCATCAGACTA 3'
RBS 71 (SEQ ID NO. 7)	5' AGCACACATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTG 3'
RBS 72 (SEQ ID NO. 8)	5' GACTTGTCGACAAAAACCCCTCAAGACCCG 3'
RBS 87 (SEQ ID NO. 9)	5' Spacer: GATCAT XhoI: CTCGAG 5' end of aerobactin promoter: CGCCATATCCTCCCAGAG 3'
RBS 88 (SEQ ID NO. 10)	5' Spacer: GATCAT BglII: AGATCT 3' end of aerobactin promoter: ACACAGTAAAATAATAAC 3'

Design for a suicide expression vector according to the invention:

A design for a preferred suicide expression vector according to the invention is shown diagrammatically in Fig. 9A TO 9F. The expression vector includes the following elements:

(i) foreign ("vaccine") antigen expressing gene cassette (including a constitutive or inducible promoter sequence),

(ii) antibiotic or other resistance or selection marker.

(iii) origin of replication.

(iv) restriction enzyme gene (e.g.. I-*Ppo*I) with an inducible gene expression promoter (e.g. T7 promoter sequence),

(v) ribozyme catalytic region,

(vi) an inducible promoter to express the ribozyme (e.g. *paer* promoter).

(vii) a transcription terminator to terminate the mRNA transcript originating from the promoter mentioned in (vi), and

(viii) one or more cloned intron-encoded restriction enzyme (e.g. I-*Ppo*I) cleavage sites.

EXAMPLE 1:- Bacterial vaccine transformed with a suicide expression vector.

A recombinant suicide expression vector of the above design, carrying gene(s) encoding vaccine antigen(s), under the control of an *in vitro* inducible promoter is transformed into a bacterial vaccine delivery vector such as *Salmonella typhimurium aro*A- by any of the methods well known to the art. The recombinant *S. typhimurium aro*A- strain is induced to activate the promoter and express high levels of the vaccine antigen(s). Prior studies on the kinetics of vaccine antigen expression in the recombinant *Salmonella* would indicate the time required to achieve maximal expression of the vaccine antigen(s). The recombinant *Salmonella* would therefore be induced for the length of time required for maximal vaccine antigen expression. The growth media may also include an inducer for the promoter sequence controlling expression of a ribozyme catalytic region (e.g. the growth media would include 2',2-dipyridyl (an iron chelator) to induce expression of ribozyme from a ribozyme catalytic region under the control of the *paer* promoter. Addition of IPTG (isopropyl-β-thiogalactopyranoside) to the growth media would induce the p*lacUV5* controlled expression of the T7 RNA

polymerase protein from a T7 RNA polymerase gene preferably present on the suicide expression vector. Induced expression of the T7 RNA polymerase would be expected to overcome the repressing effect of the ribozyme on T7 promoter driven expression of I-*Ppo*I restriction enzyme and thereby lead to the expression of I-*Ppo*I restriction enzyme to effect cleavage of the suicide expression vector. Additionally, it may be possible to repress *paer* promoter with the addition of $FeCl_3$ into the growth media. Such repression may decrease expression of the ribozyme to basal levels permitting greater I-*Ppo*I activity. The *Salmonella typhimurium aro*A- chromosome does not carry any I-*Ppo*I sites. Once cleaved, the bacterial endogenous exonucleases would be expected to degrade the resulting linear fragments of plasmid DNA.

The kinetics of the loss of the suicide expression vector from the bacterial cells can be analysed to determine the time required for complete loss of expression vector DNA from the vaccine preparation. Simultaneous analyses can also be carried out to ensure that the vaccine antigen in the absence of any further expression following addition of IPTG is sufficient to elicit an effective immune response.

The vaccine strain carrying maximal amounts of the vaccine antigen but completely devoid of recombinant expression vector DNA would then be ready for release into the environment e.g. in baits.

EXAMPLE 2:- Expression of restriction enzyme I-*Ppo*I.

Materials and methods:

Materials

Plasmid pI3-941 (Muscarella *et. al.*, 1990) with the gene for the restriction enzyme I-*Ppo*I was provided by the laboratory of Dr Vogt. Expression vector pET 21d was obtained from Novagen (Madison. WI., U.S.A.), general molecular biology reagents were sourced from Boehringer Mannheim, Promega and New England Biolabs and general laboratory reagents were from Sigma. *E. coli* strain JM109 (DE3) (Yanisch-Perron *et. al.*, 1985) was purchased from Promega Corp., (Madison. WI., U.S.A.) and was used for the transformation of plasmid constructs. The strain carries chromosomally integrated bacteriophage lambda which carries the gene encoding T7 RNA polymerase. *Salmonella typhimurium aro*A- strain H4335 (Brahmbhatt *et. al.*, 1997) was used as a vaccine carrier strain.

Synthetic oligonucleotides used in PCR reactions were synthesised by Ransom Hill Bioscience Inc., (Ramona, CA., U.S.A.) and are listed in Table1. *Pfu* turbo was obtained from Stratagene.

Methods

All DNA manipulation experiments were performed as standard molecular biology techniques (Sambrook *et.al.*, 1989).

Expression of I-*Ppo*I from the plasmid constructs was determined by growing the bacterial cells harbouring the expression plasmid to $OD_{600} = 0.5$ and inducing with 1 mM IPTG for 1 to 3 hours.

Genetic construction of ribozyme (Figure 3):

The oligos RBS 68, 69, 70 and 71 (Table 1) were pooled in equal amounts and diluted to give a final concentration of 50 ng/µl of DNA. One microlitre of this pooled DNA was used as the template for PCR. The oligos RBS 67a and 72 (Table 1) were used as the PCR primers. The reaction consisted of 0.3 mM each dNTP, 0.8 µM of each PCR primer, 50 ng of template DNA, 2.5 units of *pfu* turbo DNA polymerase and 4 mM $MgSO_4$ all in 50 µl of reaction buffer. The annealing temperature was 65°C for one minute,extension at 72°C was for 30 seconds and denaturation at 94°C for 1min. The PCR product was precipitated and digested with*Xho* I and *Sal* I and cloned into the vector pI3-941 prepared with the same enzymes. The resulting vector was called pRBS 43 and was confirmed by digestion with*Xho* I and *Sal* I together and with *Bgl* II alone.

Results:

Demonstration that I-*Ppo*I does not cleave *E. coli* & *S. typhimurium* chromosomal DNA.

Chromosomal DNA was prepared from a variety of *E. coli* and *Salmonella* strains and incubated *in vitro* with purchased enzyme I-*Ppo*I. The same DNAs were also incubated with other restriction enzymes e.g. *Eco*R I, *Not* I, *Xba* I and *Hind* III. All incubations were carried out in the provided incubation buffer. The DNAs were analysed by agarose gel electrophoresis and ethidium bromide staining (data not shown). The results clearly showed that there were no cleavage sites for I-*Ppo*I in the *E. coli* and *Salmonella* chromosomes. This is significant since if the chromosome contains the I-*Ppo*I site then the concept of suicide plasmid would not be applicable since

cytoplasmic expression of I-*Ppo*I would be deleterious to the viability of the carrier strain to be used as a vaccine or for environmental bioremediation.

Plasmid Construction and analysis of I-*Ppo*I function in recombinant *E. coli*.

Plasmid pI3-941 (Fig. 1) carries the I-*Ppo*I restriction enzyme encoding gene and the enzyme is expressed under the control of the T7 promoter. As a first step, it was necessary to determine the following:

(1) Is the eucaryotic I-*Ppo*I restriction enzyme functional in procaryotic Gram-negative bacteria such as *E. coli*.

(2) If the I-*Ppo*I enzyme does cleave plasmid DNA carrying the I-*Ppo*I recognition site, then do the endogenous *E. coli* exo- and/or endo-nucleases digest the recombinant plasmid.

(3) If (1) and (2) are achieved, then would the recombinant plasmid cleavage be sufficient to completely eliminate recombinant DNA from the bacterial cell.

To answer the above questions the following set of recombinant plasmids were constructed and analysed for self-restriction and cellular plasmid maintenance in the uninduced and induced (for I-*Ppo*I expression) state.

The I-*Ppo*I restriction enzyme cleavage site was initially cloned into plasmid pI3-941 to give plasmid pRBS 22. To accomplish this, plasmid pI3-941 was cleaved with *Xho* I, end-filled with T4 DNA polymerase, phosphatased and ligated to annealed, *Sma* I cleaved oligos RBS 3 and RBS 4. The resulting plasmid pRBS 22 (Fig. 1) carries the I-*Ppo*I restriction enzyme site harboured in the synthetic oligonucleotide insert DNA.

Both plasmids (pI3-941 and pRBS 22) were transformed into the host strain JM109 (DE3) for analysis of expression of the I-*Ppo*I enzyme from the T7 promoter. Agarose gel analysis of plasmid profiles from the recombinant strains revealed (Fig. 2) that even in the uninduced state the plasmid pRBS 22 was eliminated to such low levels that it was not readily visible by ethidium bromide staining. This data indicated that the eucaryotic I-*Ppo*I restriction enzyme is functional in the cytoplasm of procaryotic *E. coli*. The enzyme activity is very high and hence even residual (leaky) expression of I-*Ppo*I from the T7 promoter is sufficient to cleave the recombinant plasmid. The data also demonstrates that once I-*Ppo*I cleavage occurs, the *E. coli* endogenous nucleases are able to completely eliminate the recombinant plasmid.

The data indicates that stringent repression of the promoter is desirable to maintain the plasmid in the cell in the uninduced state which is required to ensure that recombinant DNA gene product(s) are expressed to high levels before elimination of recombinant DNA. Thus, with a desire to fully repress promoter expression in the uninduced plasmid, it was hypothesised that the use of a ribozyme (anti-sense catalytic RNA), expressed at low to medium levels, that targets the I-*Ppo*I mRNA, may reduce enzyme expression to a level where the plasmid is stable but still allows self-restriction upon high level induction of expression of I-*Ppo*I.

A synthetic DNA sequence was designed (Fig. 3, SEQ ID NO. 11) which carries the ribozyme catalytic DNA sequence which targets the I-*Ppo*I intron 3 sequence at position 345bp – 369bp. It also carries the I-*Ppo*I cleavage site and the ribozyme expression is under the control of the constitutive OmpA promoter. Transcription of the ribozyme is terminated at the T7 terminator. The entire gene cassette was synthesised with oligonucleotides as described in "Methods". The resulting plasmid pRBS 43 was transformed into the host strain JM109 (DE3) and tested for stability and self-restriction. Upon induction with 1 mM IPTG for 1 hour some plasmid is lost (Fig. 4). Of particular significance is that in the uninduced state there was a large amount of plasmid present which contrasts with that seen with the vector pRBS 22. This data indicates that the presence of the ribozyme does stabilise the plasmid, reducing the level of expression of the I-*Ppo*I enzyme in the uninduced state.

Two plausible reasons for the limited self-restriction of the plasmid upon induction may be:

(1) The I-*Ppo*I restriction site is adjacent to the OmpA promoter and hence the constitutive mRNA transcription from the promoter may sterically hinder the I-*Ppo*I restriction enzyme from binding to the cleavage site.

(2) The OmpA promoter being a medium-level and constitutive promoter may result in excessive levels of the ribozyme which may result in minimal quantities of I-*Ppo*I mRNA that could be translated into active enzyme.

To test the above two hypotheses, two different plasmids were constructed. To test hypothesis (1), an additional I-*Ppo*I restriction site was cloned upstream of the T7 promoter in plasmid pRBS 43 to give plasmid pRBS 45 (Fig. 1) to determine if p*Omp*A-distal location of the I-*Ppo*I site may

enhance I-*Ppo*I binding and cleavage. To accomplish this, the plasmid pRBS 43 was digested with *Ssp* I, CIP treated and ligated to the I-*Ppo*I site fragment generated by annealing and cleavage with *Sma* I of oligos RBS 3 and RBS 4 (Table 1). This resulted in plasmid pRBS 45.

For hypothesis (2), the OmpA promoter in plasmid pRBS 43 was replaced with a part of the iron regulated aerobactin promoter designated p aer (Bindereif and Neilands, 1985). This promoter is normally expressed at basal levels and is induced to higher expression under iron limiting conditions. *In-vitro*, such conditions are achieved by the addition of 200uM 2',2-Dipyridyl (iron chelator) to the growth media. This plasmid was designated pRBS 46 (Fig. 1). The cloning was carried out by PCR amplifying p*aer* from plasmid pHB170 with oligos RBS 87 and RBS 88 (Table 1), cleavage with *Bgl* II and *Xho* I and cloning the fragment into the respective sites of plasmid pRBS 43.

Both plasmids were transformed into host strain JM109 (DE3) and examined for plasmid maintenance under uninduced conditions and self-digestion following induction of I-*Ppo*I expression.

Initial experiments showed that plasmid pRBS 45 was rapidly digested even in the uninduced state suggesting that either the distal location or the additional I-*Ppo*I site on the plasmid results in rapid plasmid cleavage. It has been previously established that ribozymes are more active at higher temperature and our previous experiments had established that I-*Ppo*I activity is reduced at 42°C. An experiment was therefore conducted to determine if the plasmid could be maintained in the cell under uninduced conditions due to the partial inactivation of I-*Ppo*I and greater activity of the ribozyme. The recombinant cultures were grown initially at either 37°C or 42°C and uninduced and induced (for I-*Ppo*I expression) plasmid profiles were analysed as shown in Fig. 5. The data revealed that at 42°C, the recombinant plasmid was maintained in the cell presumably due to the combined effects of partial inactivation of I-*Ppo*I and greater activity of the ribozyme at the higher temperature. Upon induction at 37°C, a significant amount of plasmid is self-restricted. Although the data is interesting, it does not allow a practical application for the suicide plasmid in bacterial strains either for vaccines or bioremediation. The growth at 42°C is also unsuitable since prolonged growth at such higher temperatures may be detrimental for the survival of the carrier micro-organisms. For the development of strains

for bioremediation, the higher temperature may also adversely affect the activity of the enzymes used to target the toxic waste.

Plasmid cleavage of pRBS 46 (Fig. 6) was similar to that of pRBS 43 (Fig. 2) suggesting that although the p*aer* promoter (pRBS 46) can be repressed unlike the p*Ompa* (pRBS 43), both promoters express sufficient ribozyme to inhibit plasmid cleavage.

There are at least two plausible reasons for the above observations:

(1) Upstream of p*Ompa* (pRBS 43) and paer (pRBS 46), is located the strong promoter p*lac*. It may be possible that this promoter may override p*Ompa* or p*aer* thereby expressing more ribozyme.

(2) Although p*Ompa* is replaced with p*aer* in plasmid pRBS 46, the plasmid does not carry the distal I-*Ppo*I site and hence it is still possible that the p*aer*mRNA transcript may sterically hinder the binding of I-*Ppo*I to the promoter proximal I-*Ppo*I site.

To address the above two possibilities, an additional two plasmids were constructed namely pRBS 47 and pRBS 48 (Fig. 1).

To eliminate the potential over-riding effect of p*lac*, the *Xba* I / *Xho* I insert DNA in pRBS 46 was cloned into the respective sites of plasmid pET-21d to give plasmid pRBS 47. This plasmid does not carry additional promoters upstream of the *Xho* I site and hence the ribozyme should be expressed solely under p*aer*. Six independent isolates were grown and plasmid profiles from uninduced and induced (1mM IPTG for 1 hr.) recombinant cells were analysed by agarose electrophoresis (Fig. 7). The results demonstrated that the plasmid was well maintained in the cells and there was no evidence of self-restriction. This data along with that observed (described above) for plasmids pRBS 43 and pRBS 46 indicates that the strength of the promoter expressing the ribozyme is not a critical factor in achieving plasmid self-restriction.

To test hypothesis (2) described above, an additional I-*Ppo*I restriction site was cloned distal to the promoter-proximal site. This was achieved by annealing oligonucleotides RBS 3 and RBS 4 (Table 1) (generates an I-*Ppo*I site), cleaving with *Sma* I and cloning it into the unique *Psh*A1 (Fig. 1) site of plasmid pRBS 47 to result in plasmid pRBS 48 (Fig. 1). Six independent isolates were grown and plasmid profiles from uninduced and induced (1mM IPTG for 1 hr.) recombinant cells were analysed by agarose electrophoresis (Fig. 8). The results clearly demonstrate effective plasmid maintenance in

uninduced cells and almost complete plasmid self-restriction upon induction of I-*Ppo*I expression.

The above data establishes the viability and utility of the suicide expression vector of the present invention, by showing that:

(1) The eucaryotic restriction enzyme I-*Ppo*I does not cleave *E. coli* and *Salmonella typhimurium* chromosomes hence such an enzyme if expressed in the bacterial cytoplasm will not be deleterious to the viability of the bacterial cell.

(2) I-*Ppo*I can be expressed in the bacterial cytoplasm using either the T7 promoter or ideally any other repressible bacterial promoter.

(3) I-*Ppo*I is active in the procaryotic bacterial cell and is able to cleave recombinant plasmid harboured I-*Ppo*I restriction site(s).

(4) Once I-*Ppo*I cleaves the recombinant plasmid endogenous bacterial nucleases rapidly eliminate the plasmid DNA.

(5) Since most bacterial promoters are "leaky" for expression. the ribozyme technology can be used to cleave I-*Ppo*I mRNA under non-induced conditions. This property is useful for plasmid maintenance in the cell during the process of plasmid-borne recombinant foreign antigen expression.

(6) The ribozyme can be expressed using an inducible promoter like p*aer* since it may be possible to modulate the amount of ribozyme produced.

(7) The I-*Ppo*I site should ideally be located at a site distal to any promoter since promoter transcription may sterically hinder I-*Ppo*I binding to the recognition site.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are. therefore, to be considered in all respects as illustrative and not restrictive.

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SEQUENCE LISTING

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The Australian National University
Agriculture Protection Board of Western Australia and
Department of Conservation and Land Management

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Claims:

1. A suicide expression vector for expressing a heterologous peptide, polypeptide or protein in a selected host cell, said vector comprising:
(i) a first nucleotide sequence encoding said heterologous peptide, polypeptide or protein operably linked to a first promoter sequence,
(ii) a second nucleotide sequence encoding a restriction enzyme or functional portion thereof operably linked to a second promoter sequence, said second promoter sequence being inducible, and
(iii) one or more cleavage site(s) for said restriction enzyme or functional portion thereof, said cleavage site(s) being absent from the chromosomal DNA of said host cell,
wherein upon introduction of the vector into said host cell, induced expression of the restriction enzyme or functional portion thereof from said second nucleotide sequence brings about the cleavage of the suicide expression vector.

2. A vector according to claim 1, wherein the first nucleotide sequence encodes an antigen, enzyme or toxin.

3. A vector according to claim 2, wherein the first nucleotide sequence encodes a contraceptive antigen.

4. A vector according to claim 2, wherein the first nucleotide sequence encodes an esterase capable of hydrolysing organophosphates.

5. A vector according to claim 2, wherein the first nucleotide sequence encodes an insecticidal toxin.

6. A vector according to any one of the preceding claims, wherein the second nucleotide sequence encodes a restriction enzyme or functional portion thereof that recognise a cleavage site(s) of ten or more nucleotides.

7. A vector according to claim 6, wherein the second nucleotide sequence encodes a restriction enzyme selected from the group consisting of I-*Ppo*I, I-*Ceu*I, P1-*Psp*I, P1-*T1i*I and P1-*Sce*I.

8. A vector according to any one of the preceding claims, wherein the one or more cleavage site(s) is/are located at a site(s) on the vector which avoids steric hindrance of binding by said restriction enzyme or functional portion thereof.

9. A vector according to any one of the preceding claims, further comprising a third nucleotide sequence encoding a ribozyme targetted against mRNA produced from the said second nucleotide sequence encoding the restriction enzyme or functional portion thereof.

10. A vector according to any one of the preceding claims, wherein the second promoter is selected from the group consisting of the p*lacZ* promoter, the p*lacUV5* promoter and the T7 RNA polymerase promoter.

11. A vector according to claim 10, wherein the second promoter is the T7 RNA polymerase promoter.

12. A vector according to claim 11, further comprising an additional nucleotide sequence encoding T7 RNA polymerase operably linked to a third promoter sequence, said third promoter sequence being inducible.

13. A host cell transformed with a suicide expression vector according to any one of the preceding claims.

14. A host cell according to claim 13, wherein said host cell is a bacterium or yeast.

15. A method of expressing a heterologous peptide, polypeptide or protein in a selected host cell, comprising;
(i) transforming said host cell with a suicide expression vector according to any one of claims 1 to 12,
(ii) culturing said transformed host cell under suitable conditions for the expression of the said heterologous peptide, polypeptide or protein, and
(iii) thereafter inducing expression of the restriction enzyme or functional portion thereof to bring about cleavage of the said suicide expression vector.

16. A method according to claim 15, wherein the host cell is a bacterium or yeast.

17. A method for the production of a microorganism vector which contains recombinant peptide, polypeptide or protein but no recombinant DNA, comprising;
(i) transforming said microorganism with a suicide expression vector according to any one of claims 1 to 12,
(ii) culturing said transformed microorganism under suitable conditions for the expression of the said heterologous peptide, polypeptide or protein, and
(iii) thereafter inducing expression of the restriction enzyme or functional portion thereof to bring about cleavage of the said suicide expression vector.

18. A method according to claim 17, wherein the microorganism is a bacterium or yeast.

19. A microorganism vector produced by the method according to claim 17 or 18.

FIGURE 1

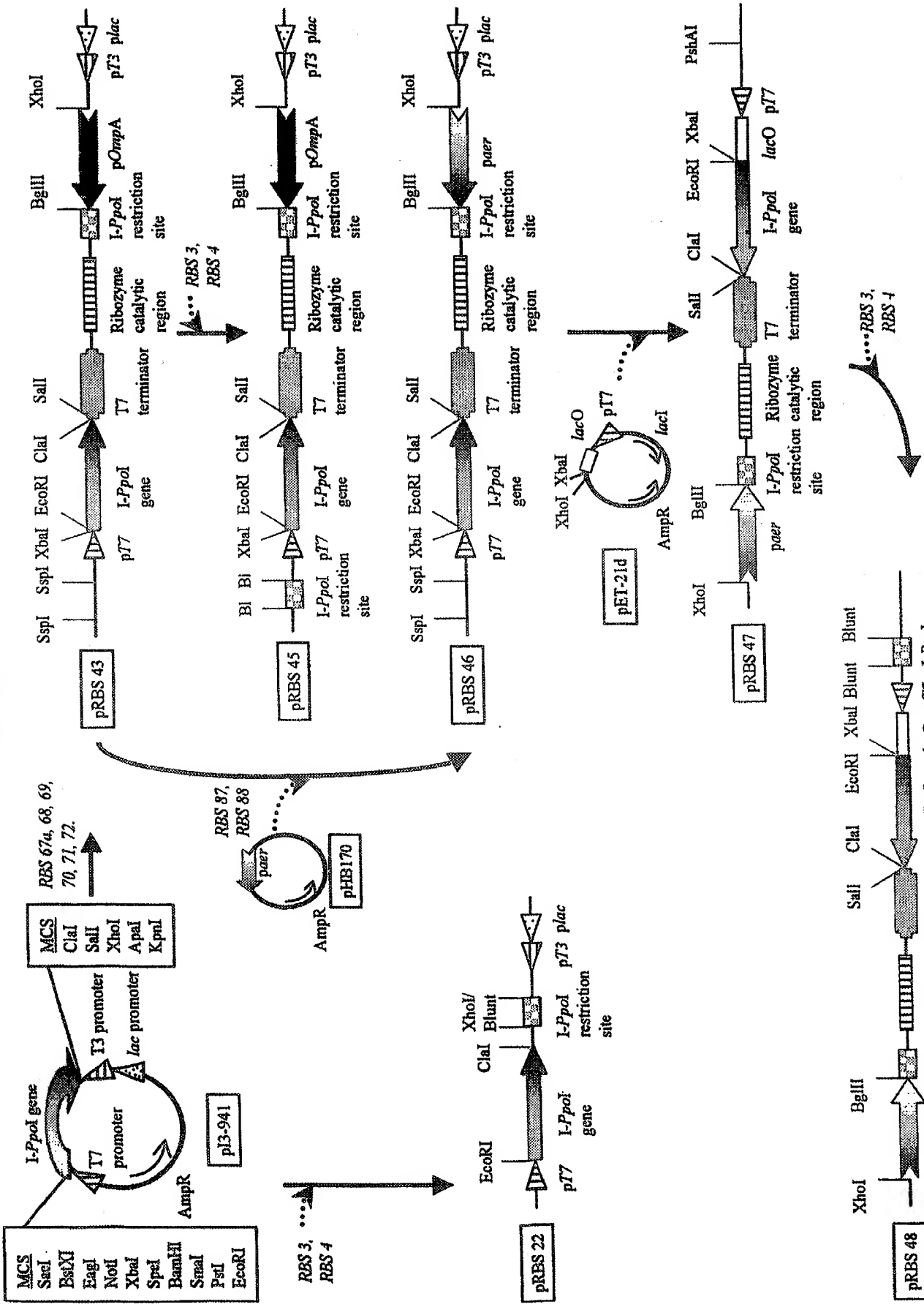

MCS
SacI
BstXI
EagI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI
I-PpoI gene
T7 promoter
T3 promoter
lac promoter
AmpR
pI3-941
RBS 674, 68, 69, 70, 71, 72.
MCS
ClaI
SalI
XhoI
ApaI
KpnI
RBS 87, RBS 88
paer
AmpR
pHB170
RBS 3, RBS 4
pRBS 22
EcoRI
I-PpoI gene
pT7
ClaI
XhoI/ Blunt
I-PpoI restriction site
pT3 plac
pRBS 43
SspI
SspI
XbaI
EcoRI
ClaI
SalI
pT7
I-PpoI gene
T7 terminator
Ribozyme catalytic region
BglII
I-PpoI restriction site
pOmpA
pT3 plac
XhoI
RBS 3, RBS 4
pRBS 45
BI
BI
XbaI
EcoRI
ClaI
SalI
I-PpoI restriction site
pT7
I-PpoI gene
T7 terminator
Ribozyme catalytic region
BglII
I-PpoI restriction site
pOmpA
pT3 plac
XhoI
pRBS 46
SspI
SspI
XbaI
EcoRI
ClaI
SalI
pT7
I-PpoI gene
T7 terminator
Ribozyme catalytic region
BglII
I-PpoI restriction site
paer
pT3 plac
XhoI
pET-21d
XhoI
XbaI
lacO
pT7
lacI
AmpR
pRBS 47
XhoI
BglII
paer
I-PpoI restriction site
Ribozyme catalytic region
T7 terminator
SalI
ClaI
EcoRI
XbaI
lacO
pT7
I-PpoI gene
PshAI
RBS 3, RBS 4
pRBS 48
XhoI
BglII
paer
I-PpoI restriction site
Ribozyme catalytic region
T7 terminator
SalI
ClaI
EcoRI
XbaI
Blunt
lacO
pT7
I-PpoI gene
XbaI
Blunt
pT7
I-PpoI restriction site

FIGURE 2

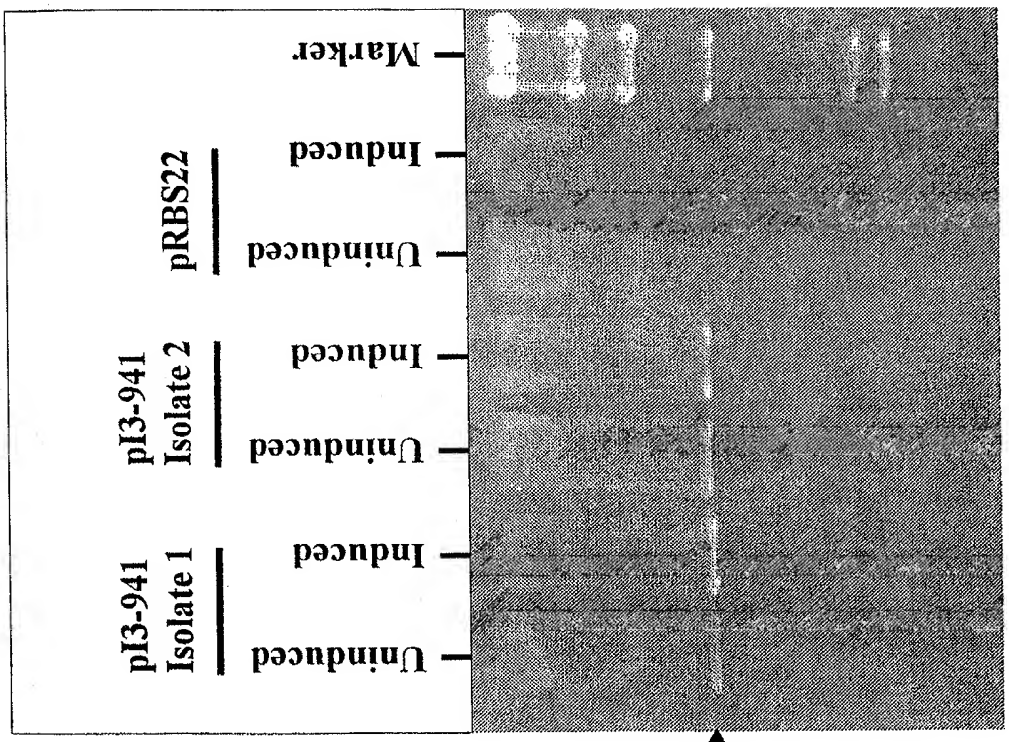

Marker
Induced
Uninduced
pRBS22
Induced
Uninduced
pI3-941 Isolate 2
Induced
Uninduced
pI3-941 Isolate 1
Recombinant Plasmid

FIGURE 3

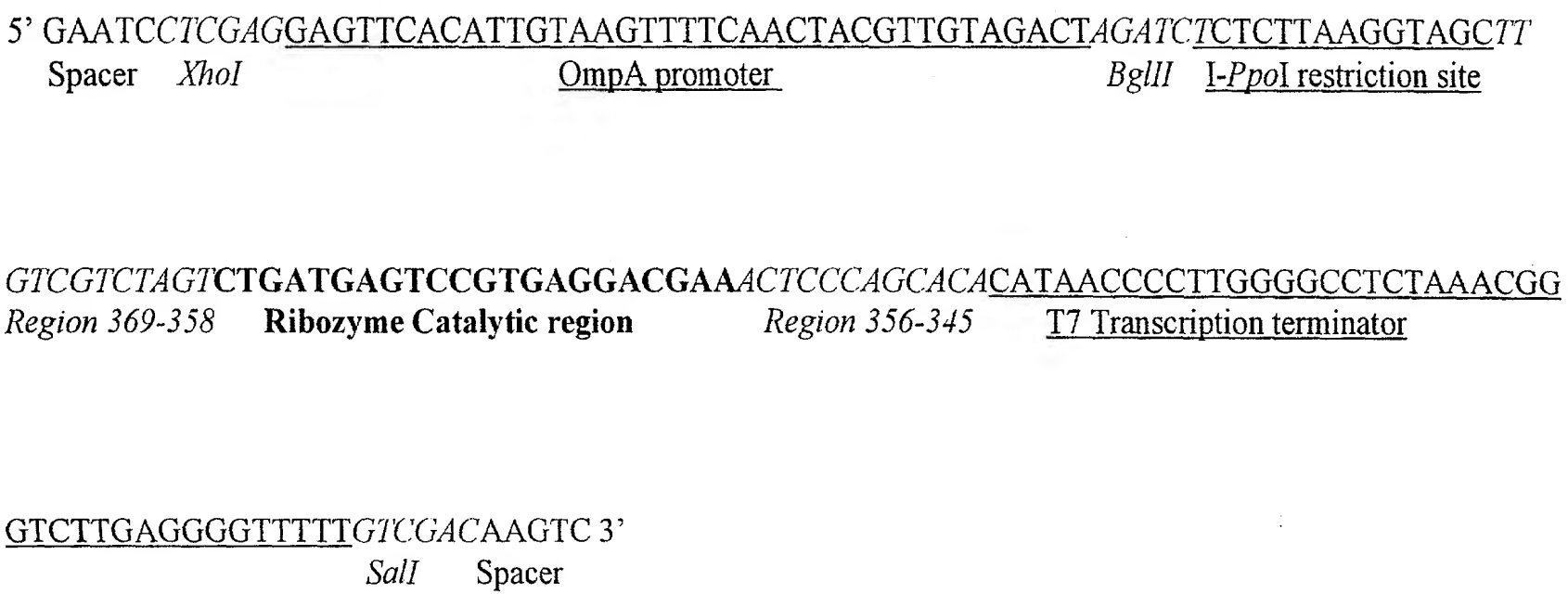

5' GAATCCTCGAGGAGTTCACATTGTAAGTTTTCAACTACGTTGTAGACTAGATCTCTCTTAAGGTAGCTT
Spacer XhoI OmpA promoter BglII I-PpoI restriction site
GTCGTCTAGTCTGATGAGTCCGTGAGGACGAAACTCCCAGCACACATAACCCCTTGGGGCCTCTAAACGG
Region 369-358 Ribozyme Catalytic region Region 356-345 T7 Transcription terminator
GTCTTGAGGGGTTTTTGTCGACAAGTC 3'
SalI Spacer

FIGURE 4

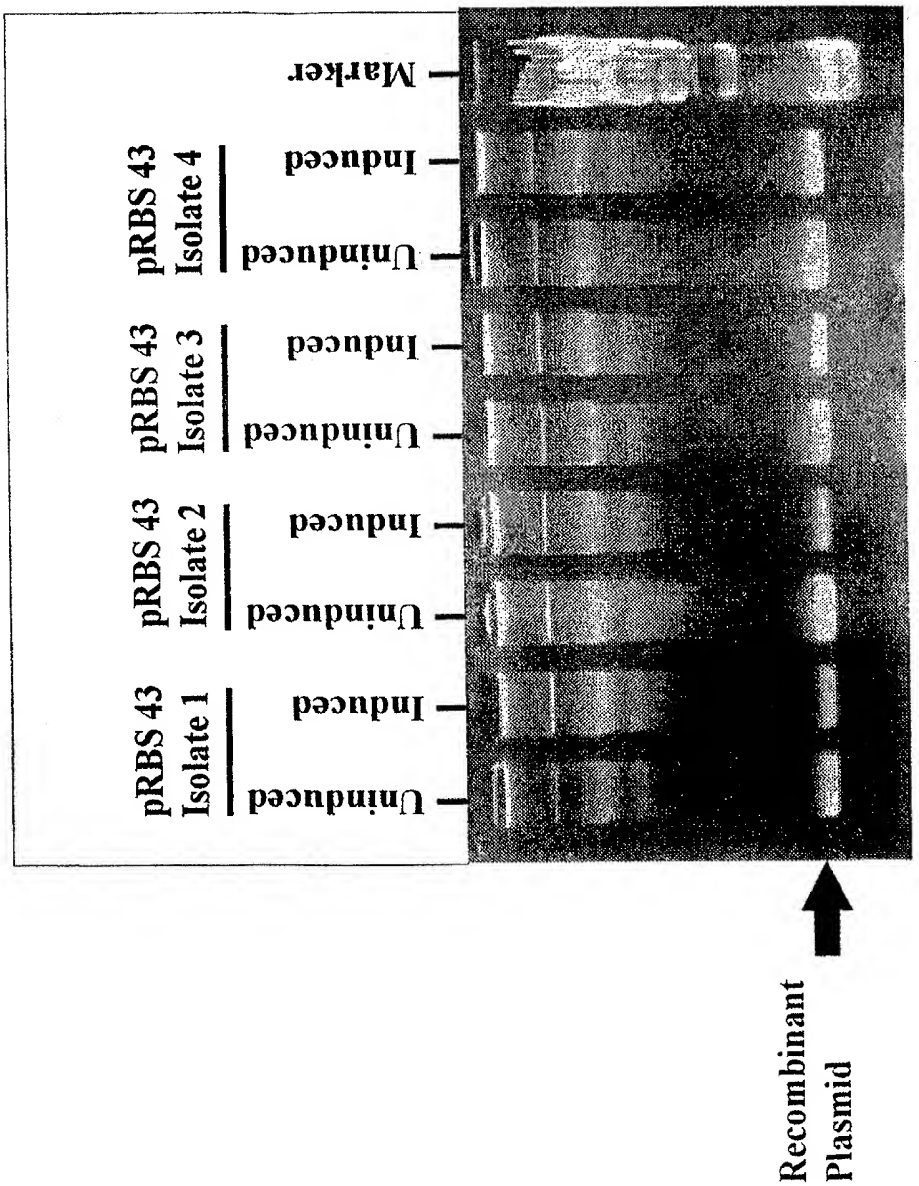

Marker
pRBS 43 Isolate 4
Induced
Uninduced
pRBS 43 Isolate 3
Induced
Uninduced
pRBS 43 Isolate 2
Induced
Uninduced
pRBS 43 Isolate 1
Induced
Uninduced
Recombinant Plasmid

FIGURE 5

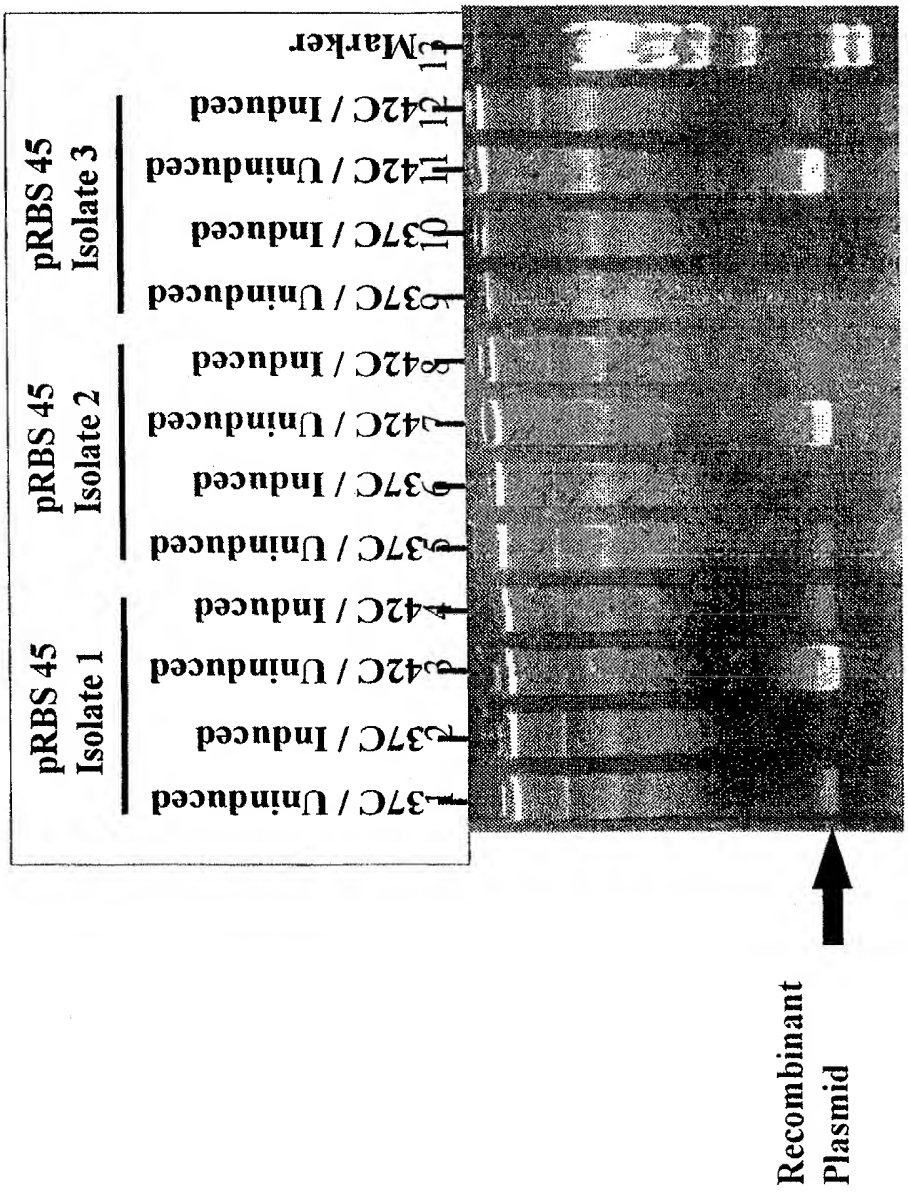

pRBS 45 Isolate 1
pRBS 45 Isolate 2
pRBS 45 Isolate 3
37C / Uninduced
42C / Induced
37C / Induced
42C / Uninduced
37C / Uninduced
42C / Induced
37C / Induced
42C / Uninduced
37C / Uninduced
42C / Induced
37C / Induced
42C / Uninduced
Marker
Recombinant Plasmid

FIGURE 6

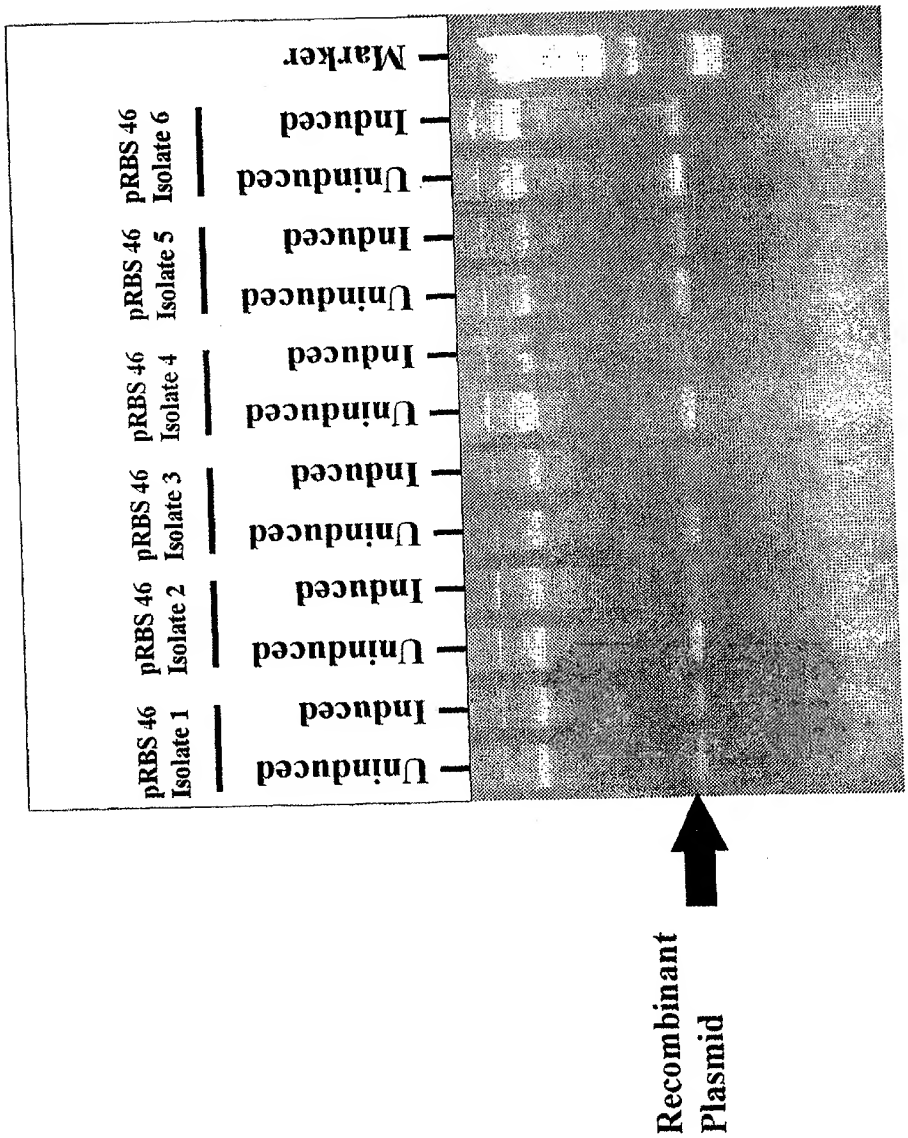

Marker
pRBS 46 Isolate 6
Induced
Uninduced
pRBS 46 Isolate 5
Induced
Uninduced
pRBS 46 Isolate 4
Induced
Uninduced
pRBS 46 Isolate 3
Induced
Uninduced
pRBS 46 Isolate 2
Induced
Uninduced
pRBS 46 Isolate 1
Induced
Uninduced
Recombinant Plasmid

FIGURE 7

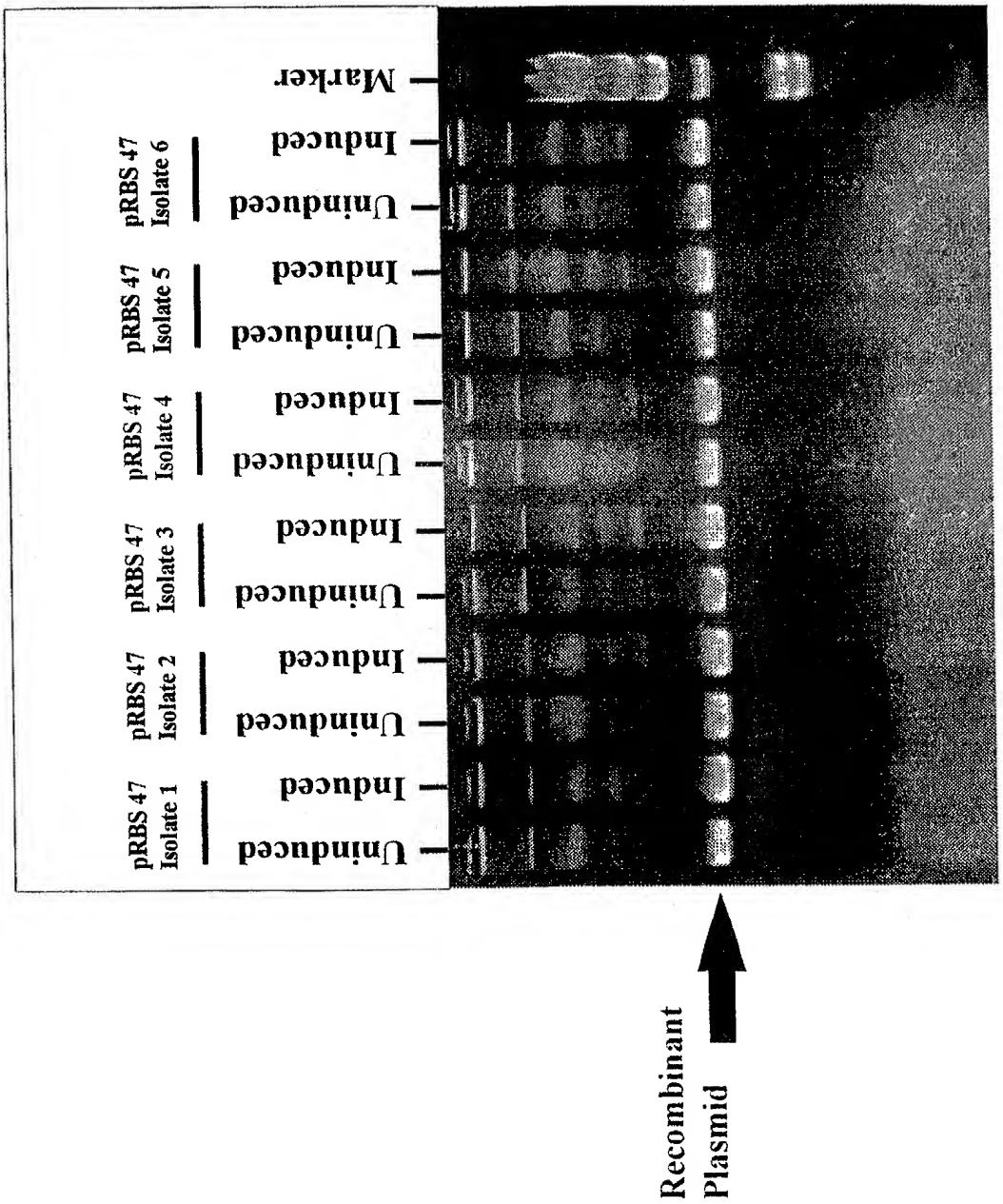

Uninduced
Induced
pRBS 47 Isolate 1
Uninduced
Induced
pRBS 47 Isolate 2
Uninduced
Induced
pRBS 47 Isolate 3
Uninduced
Induced
pRBS 47 Isolate 4
Uninduced
Induced
pRBS 47 Isolate 5
Uninduced
Induced
pRBS 47 Isolate 6
Marker
Recombinant Plasmid

FIGURE 8

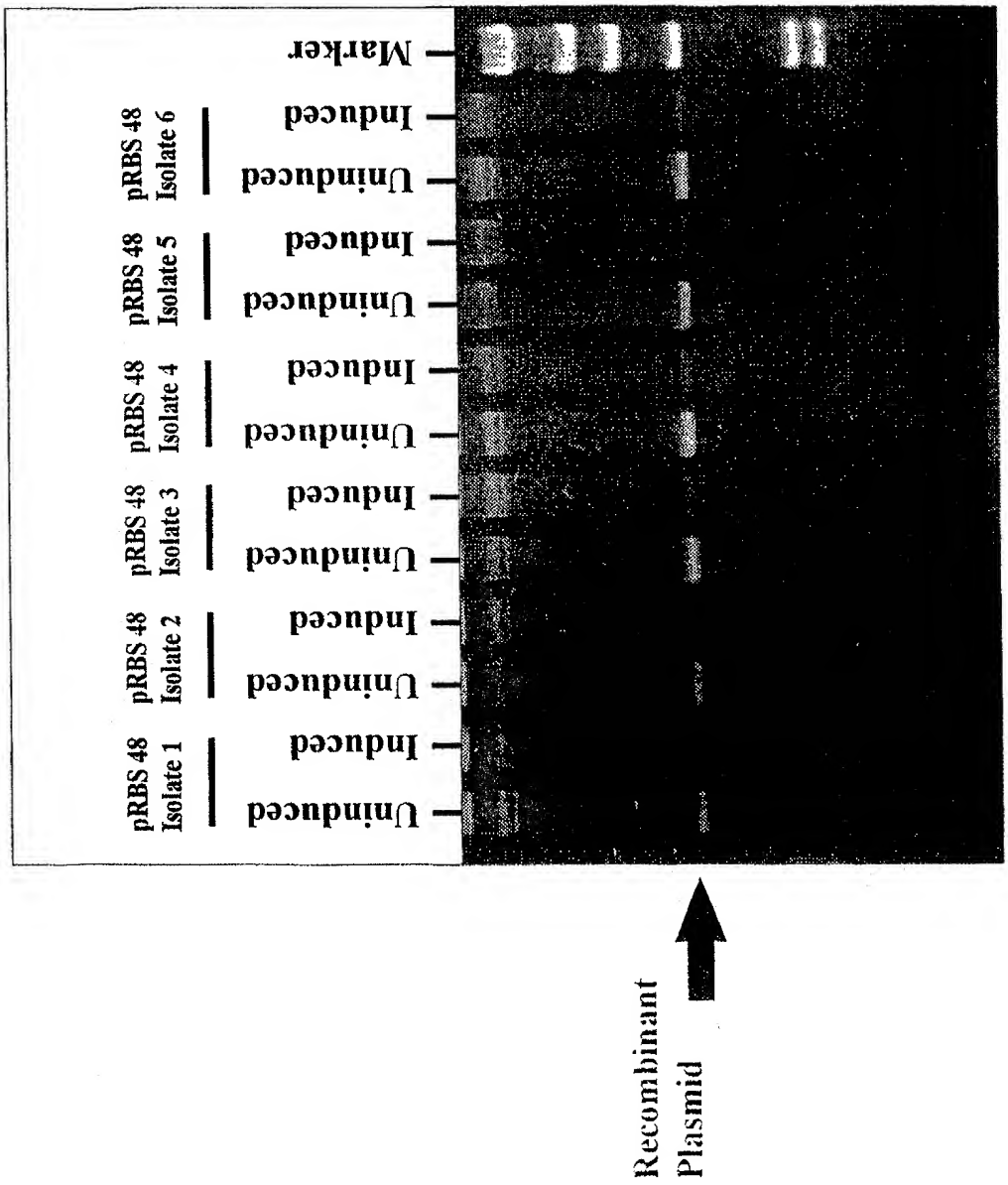

Uninduced
Induced
pRBS 48 Isolate 1
Uninduced
Induced
pRBS 48 Isolate 2
Uninduced
Induced
pRBS 48 Isolate 3
Uninduced
Induced
pRBS 48 Isolate 4
Uninduced
Induced
pRBS 48 Isolate 5
Uninduced
Induced
pRBS 48 Isolate 6
Marker
Recombinant Plasmid

FIGURE 9

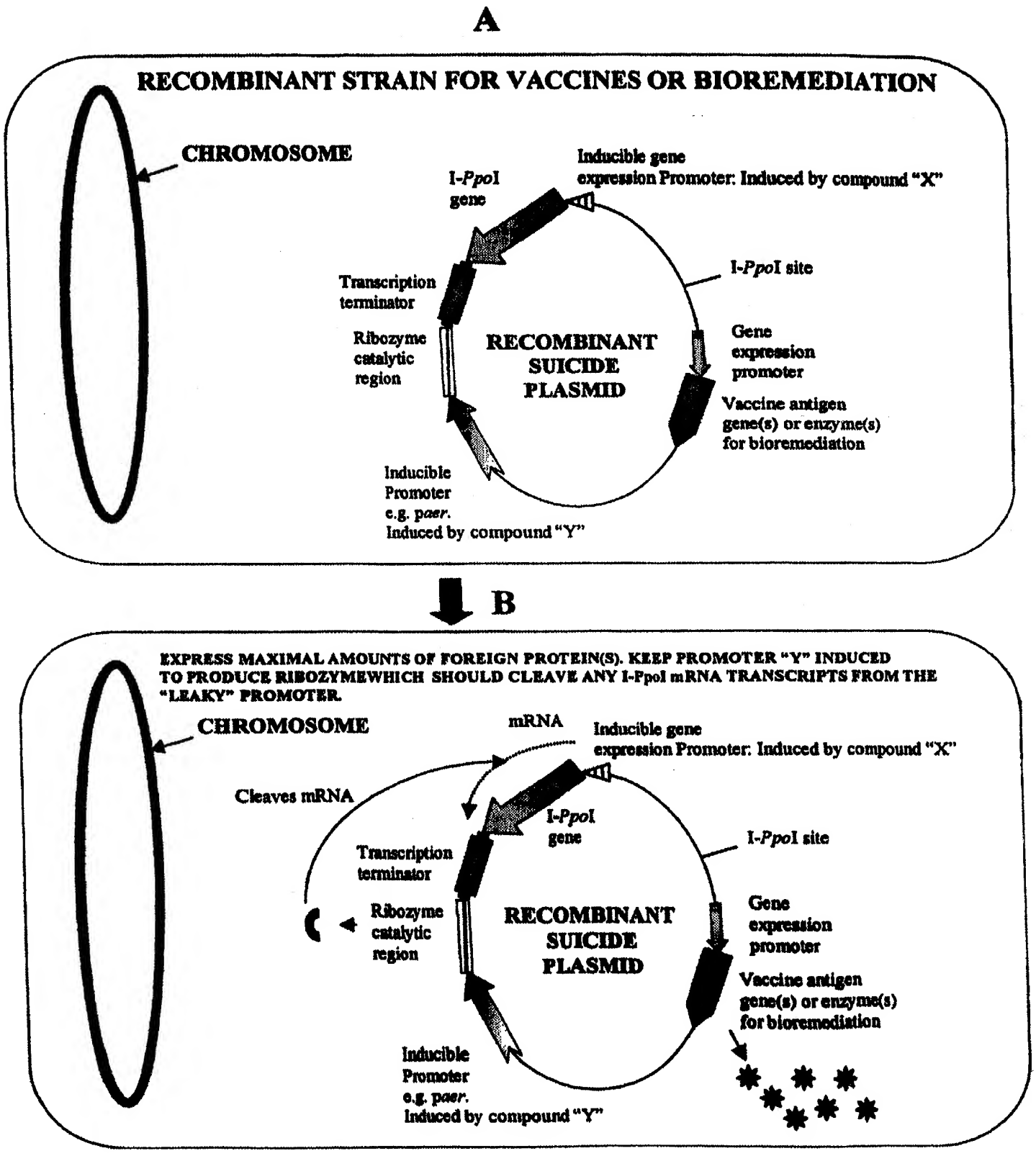
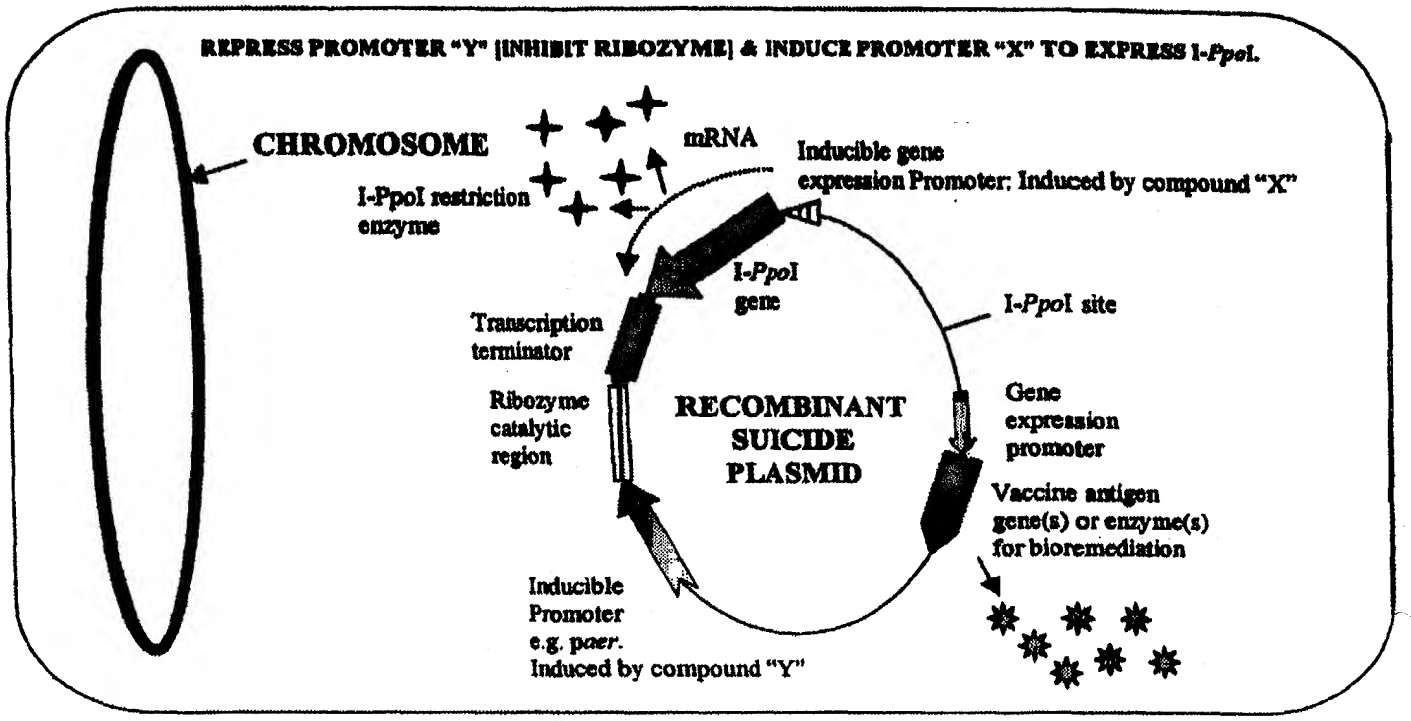

A
RECOMBINANT STRAIN FOR VACCINES OR BIOREMEDIATION
CHROMOSOME
I-PpoI gene
Inducible gene expression Promoter: Induced by compound "X"
I-PpoI site
Transcription terminator
Ribozyme catalytic region
RECOMBINANT SUICIDE PLASMID
Gene expression promoter
Vaccine antigen gene(s) or enzyme(s) for bioremediation
Inducible Promoter e.g. paer. Induced by compound "Y"
B
EXPRESS MAXIMAL AMOUNTS OF FOREIGN PROTEIN(S). KEEP PROMOTER "Y" INDUCED TO PRODUCE RIBOZYMEWHICH SHOULD CLEAVE ANY I-PpoI mRNA TRANSCRIPTS FROM THE "LEAKY" PROMOTER.
CHROMOSOME
mRNA
Inducible gene expression Promoter: Induced by compound "X"
Cleaves mRNA
I-PpoI gene
I-PpoI site
Transcription terminator
Ribozyme catalytic region
RECOMBINANT SUICIDE PLASMID
Gene expression promoter
Vaccine antigen gene(s) or enzyme(s) for bioremediation
Inducible Promoter e.g. paer. Induced by compound "Y"

FIGURE 9-CONTINUED

C



D

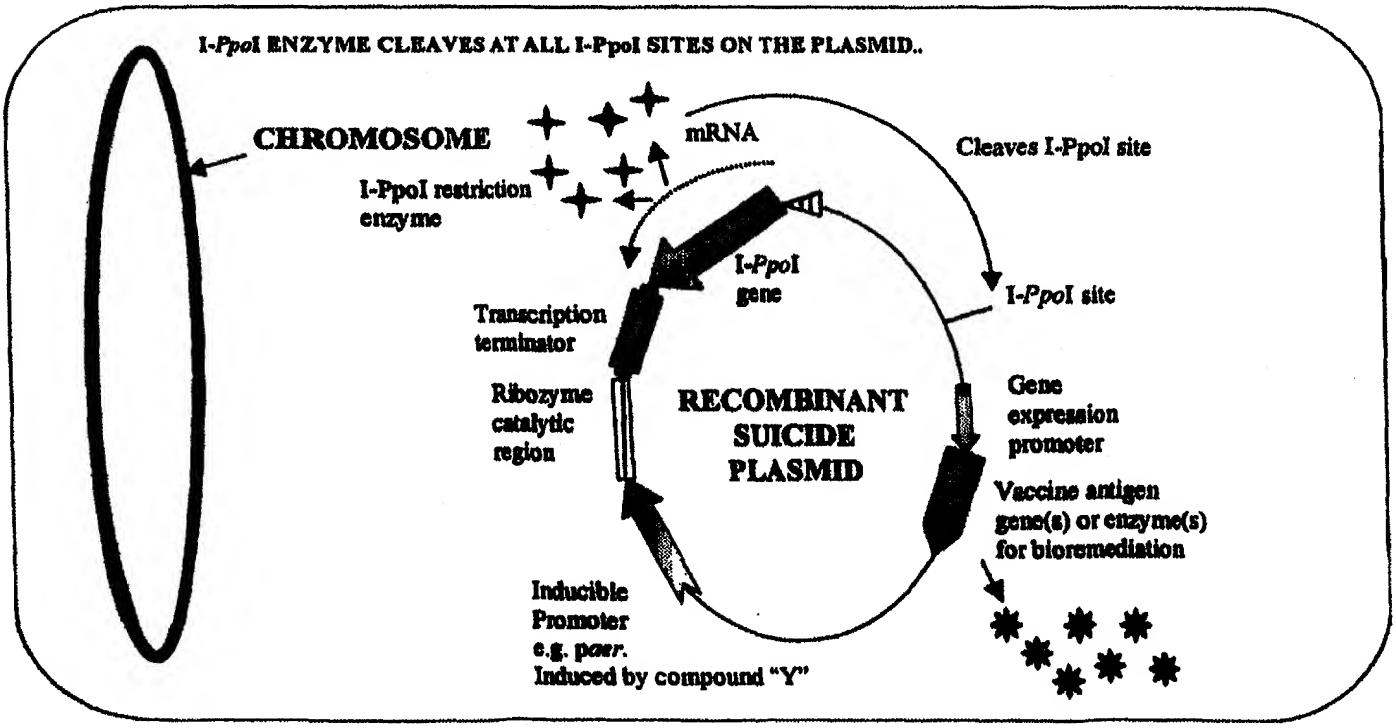
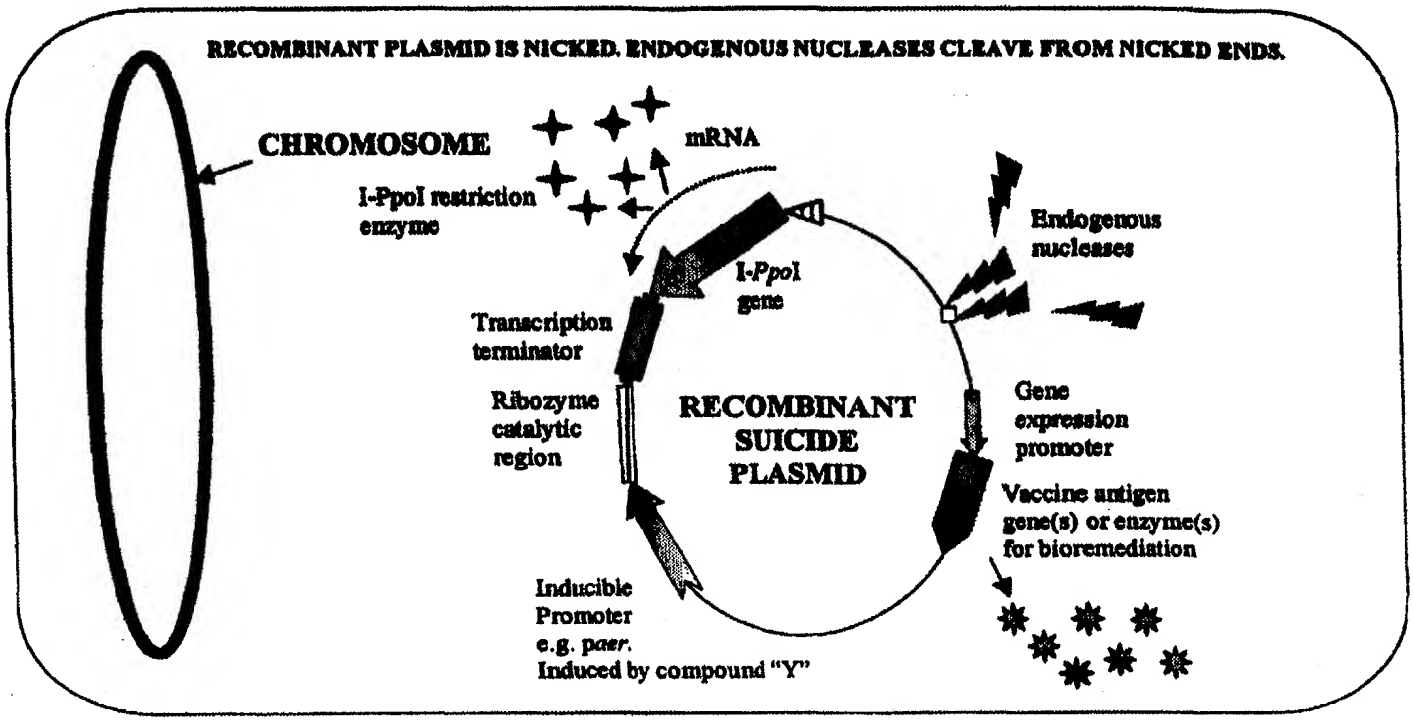
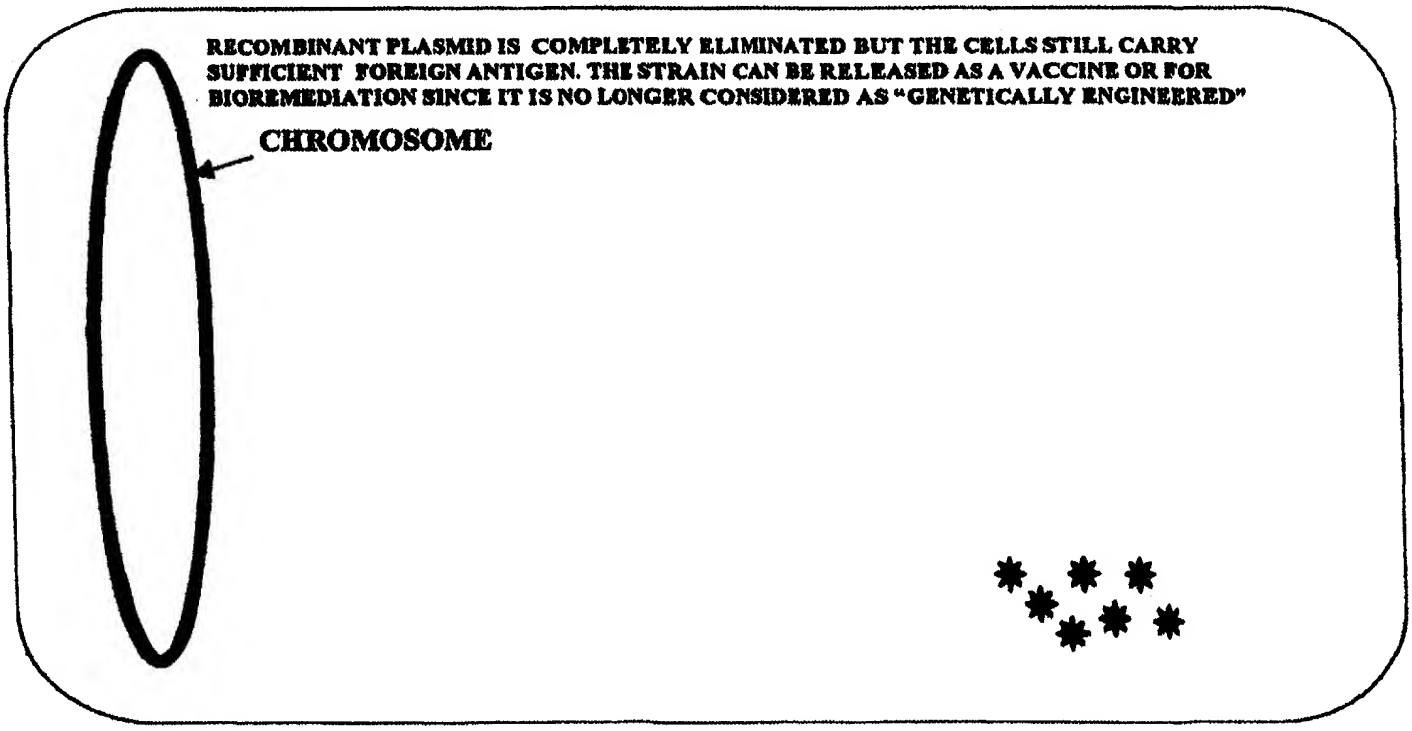


FIGURE 9-CONTINUED

E



F



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00930

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/63 - 15/86 + SUICID: OR DEATH OR KILL:

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: Keywords as above
Chemabs: Plasmid and episome/ct

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A, 9534643 (WACKERNAGEL W) 21 December 1995	
A	Appl. Environ. Microbiol., v 60, no. 10, Oct 1994, pp 3746-51, Ahrenholtz I *et al*, "A conditional suicide system in Escherichia coli based on the intracellular degradation of DNA"	
A	Curr. Opin. Biotechnol., v 4, no. 3, Jun 1993, pp 299-305, Molin S, "Environmental potential of suicide genes"	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1998	16 DEC 1998

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	**BARRY SPENCER** Telephone No.: (06) 283 2284

International Application No.
PCT/AU 98/00930

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Gene, v 118, no. 1, 1992, pp 145-6, Penfold R *et al*, "An improved suicide vector for construction of chromosomal insertion mutations in bacteria"	
A	Gene, v 29, no. 1-2, 1984, pp 63-8, Kovacs B *et al*, "The generation of a single nick per plasmid molecule using restriction endonucleases with multiple recognition sites"	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 98/00930

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	9534643	EP	717775

END OF ANNEX

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 91357	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International application No. **PCT/AU 98/00930**	International filing date *(day/month/year)* 6 November 1998	Priority Date *(day/month/year)* 6 November 1997

International Patent Classification (IPC) or national classification and IPC

Int. Cl.[6] C12N 15/63

Applicant

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand	Date of completion of the report
19 April 1999	3 August 1999
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (02) 6285 3929	Authorized Officer **PHILIPPA WYRDEMAN** Telephone No. (02) 6283 2554

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/AU 98/00930

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , filed with the letter of .

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages

[ ] the claims, Nos.

[ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	**1-19**	**YES**
	Claims	**None**	**NO**
Inventive step (IS)	Claims	**1-19**	**YES**
	Claims	**None**	**NO**
Industrial applicability (IA)	Claims	**1-19**	**YES**
	Claims	**None**	**NO**

2. Citations and explanations (Rule 70.7)

The invention as defined by claims 1-19 resides in the combination of a heterologous peptide linked to a first promoter, a restriction enzyme linked to an inducible promoter, and a cleavage site for said restriction enzyme which is absent from the chromosomal DNA of the host cell. The heterologous peptide, when introduced into a host cell, will be expressed until the expression of the restriction enzyme is induced and the cleavage of the suicide expression vector is brought about.

All of citations are directed towards killing the host cell when the suicide gene is expressed rather than destroying only the vector per se. Therefore, the invention defined by claims 1-19 is taken to be novel, involve and inventive step over the prior art and have industrial applicability.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing: 20 May 1999 (20.05.99)	
International application No.: PCT/AU98/00930	Applicant's or agent's file reference: 91357
International filing date: 06 November 1998 (06.11.98)	Priority date: 06 November 1997 (06.11.97)
Applicant: BRAHMBHATT, Himanshu, N. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

19 April 1999 (19.04.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 91357	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. **PCT/AU 98/00930**	International filing date *(day/month/year)* 6 November 1998	(Earliest) Priority Date *(day/month/year)* 6 November 1997

Applicant

(1) COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION *et al*

(2) BRAHMBHATT, Himanshu N *et al*

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of **4** sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I)

2. [ ] **Unity of invention is lacking** (See Box II)

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

   - [ ] filed with the international application
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed
   - [ ] transcribed by this Authority

4. With regard to the **title,**

   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. **9**

   - [ ] as suggested by the applicant.
   - [X] because the applicant failed to suggest a figure
   - [ ] because this figure better characterises the invention
   - [ ] None of the figures

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00930

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/63 - 15/86 + SUICID: OR DEATH OR KILL:

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: Keywords as above
Chemabs: Plasmid and episome/ct

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A, 9534643 (WACKERNAGEL W) 21 December 1995	
A	Appl. Environ. Microbiol., v 60, no. 10, Oct 1994, pp 3746-51, Ahrenholtz I *et al*, "A conditional suicide system in Escherichia coli based on the intracellular degradation of DNA"	
A	Curr. Opin. Biotechnol., v 4, no. 3, Jun 1993, pp 299-305, Molin S, "Environmental potential of suicide genes"	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1998	16 DEC 1998
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **BARRY SPENCER** Telephone No.: (06) 283 2284

International Application No.
PCT/AU 98/00930

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Gene, v 118, no. 1, 1992, pp 145-6, Penfold R *et al*, "An improved suicide vector for construction of chromosomal insertion mutations in bacteria"	
A	Gene, v 29, no. 1-2, 1984, pp 63-8, Kovacs B *et al*, "The generation of a single nick per plasmid molecule using restriction endonucleases with multiple recognition sites"	

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00930

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	9534643	EP	717775

END OF ANNEX

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6: C12N 9/22, 15/64, 15/70 // 1/21, C12R 1:19 — A1

(11) International Publication Number: **WO 95/34643**

(43) International Publication Date: 21 December 1995 (21.12.95)

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(22) International Filing Date: 9 June 1995 (09.06.95)

(30) Priority Data:
94108958.3 10 June 1994 (10.06.94) EP
(34) Countries for which the regional or international application was filed: AT et al.

(71)(72) Applicant and Inventor: WACKERNAGEL, Wilfried [DE/DE]; Carl von Ossietzky Universität, Carl-von-Ossietzky-Strasse, D-26129 Oldenburg (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LORENZ, Michael, G. [DE/DE]; Carl von Ossietzky Universität, Carl-von-Ossietzky-Strasse, D-26129 Oldenburg (DE). AHRENHOLZ, Ingrid [DE/DE]; Carl von Ossietzky Universität, Carl-von-Ossietzky-Strasse, D-26129 Oldenburg (DE). JEKEL, Manfred [DE/DE]; Carl von Ossietzky Universität, Carl-von-Ossietzky-Strasse, D-26129 Oldenburg (DE).

(74) Agents: BOETERS, Hans, D. et al.; Boeters & Bauer, Bereiteranger 15, D-81541 München (DE).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CONDITIONAL SUICIDE CELLS OF E. COLI ETC.

(57) *Abstract*

The potential risks associated with the intentional or unintentional release of genetically engineered microorganisms led to the construction of biological containment systems by which bacteria are killed in a controlled suicide process. In previously published suicide systems cell killing was caused by proteins destroying the cell membrane or cell wall. Here a conditional cell-killing system is presented which is based on the intracellular degradation of cellular DNA. The nuclease gene used was that of the extracellular nuclease of *Serratia marcescens*. The nuclease gene was deleted for the leader-coding sequence and the truncated gene was put under the control of the lambda P_L promoter. Following thermoinduction of the nuclease gene cassette in *Escherichia coli*, the cell survival dropped to 2×10^{-5}, and more than 80 % of the radioactivity labelled DNA was converted to acid soluble material within 2.5 h in the absence of cell lysis. Cells from the majority (84 %) of clones which survived thermoinduced killing turned out to be as sensitive to a second thermoinduction as the original strain. Cells of the other clones showed a somewhat slower killing kinetics or a slightly higher final level of survivors. The suicide system described combines the regulated killing of cells with the abortion of horizontal gene transfer processes by destroying DNA otherwise potentially available for conjugation, transduction and genetic transformation.

FT PLASMID AND EPISOME

(L)

SUICID? / RESTRICTION ENZYME

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? NUCLEASE

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 95/02245

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/22 C12N15/64 C12N15/70 //C12N1/21,C12R1:19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANNUAL REVIEW OF MICROBIOL., vol. 47, 1993 ANNUAL REVIEWS INC. PALO ALTO, CALIFORNIA, US, pages 139-166, S. MOLIN ET AL. 'Suicidal genetic elements an dtheir use in biological containment of bacteria' cited in the application see page 152, line 36 - page 153, line 9 see page 148, line 17 - page 150, line 2 --- -/--	1-5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 1995	10.10.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	APPLIED MICROBIOL. AND BIOTECHNOL., vol. 59, no. 5, May 1993 SPRINGER INTERNATIONAL, NEW YORK, US, pages 1361-1366, G. RECORBET ET AL. 'Conditional suicide system of Escherichia coli released into soil thet uses the bacillus subtilis sacB gene' cited in the application the whole document	1-6
A	APPLIED MICROBIOL. AND BIOTECHNOL., vol. 38, no. 1, October 1992 SPRINGER INTERNATIONAL, NEW YORK, US, pages 91-93, T. SCHWEDER ET AL. 'An expression vector system providing plasmid stability and conditional suicide of plasmid-containing cells' cited in the application the whole document	1-6
A	APPLIED AND ENVIRONMENTAL MICROBIOL., vol. 57, no. 1, January 1991 AM. SOC. MICROBIOL.,WASHINGTON, DC, US, pages 85-92, S.M. KNUDSEN AND O.H. KARLSTRÖM 'Development of efficient mechanisms for biological containment of bacteria' cited in the application the whole document	1-6
A	CARLSBERG RES. COMMUN., vol. 54, 1989 SPRINGER-VERLAG, BERLIN, BRD, pages 17-27, K. BIEDERMANN ET AL. 'Purification and characterization of a Serratia marcescens nuclease produced by Escherichia coli' cited in the application the whole document	1-6
A	WO,A,92 14819 (DU PONT) 3 September 1992 the whole document	1-6
A	EP,A,0 255 755 (BAYLOR COLLEGE MEDICINE) 10 February 1988 the whole document	1-6
A	WO,A,87 05932 (GENEXPRESS APS) 8 October 1987 the whole document	1-6

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INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/EP 95/02245

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	APPLIED AND ENVIRONMENTAL MICROBIOL., vol. 60, no. 10, October 1994 AM. SOC. MICROBIOL.,WASHINGTON, DC, US, pages 3746-3751, I. AHRENHOLTZ ET AL. 'A conditional suicide system in Escherichia coli based on the intracellular degradation of DNA' the whole document	1-6

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/EP 95/02245

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9214819	03-09-92	US-A-	5300431	05-04-94
EP-A-0255755	10-02-88	AU-A-	7362287	10-12-87
		JP-A-	63099020	30-04-88
WO-A-8705932	08-10-87	AT-T-	107697	15-07-94
		AU-A-	7232087	20-10-87
		AU-B-	646467	24-02-94
		AU-A-	7818391	14-11-91
		DE-D-	3750125	28-07-94
		DE-T-	3750125	27-10-94
		EP-A-	0273040	06-07-88
		JP-T-	1500002	12-01-89

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IC ICM C12N009-22
ICS C12N015-64; C12N015-70
ICA C12N001-21
ICI C12R001-19
CC 3-2 (Biochemical Genetics)
Section cross-reference(s): 10
TI Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temperature-sensitive promoter
ST nuclease gene expression controlled suicide Escherichia
IT Escherichia coli
Plasmid and Episome
(Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)
IT Serratia marcescens
(extracellular nuclease gene; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)
IT Deoxyribonucleic acids
RL: BPR (Biological process); BUU (Biological use, unclassified); BIOL (Biological study); PROC (Process); USES (Uses)
(intracellular DNA degrdn.; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)
IT Plasmid and Episome
(pAH12; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)
IT Plasmid and Episome
(pET81F+; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)
IT Plasmid and Episome
(pNuc4; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)
IT Plasmid and Episome
(pSF1; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)
IT Virus, bacterial
(T7, Phi10 promoter; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)

IT Death
(cell, suicide; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)

IT Virus, bacterial
(lambda, PL promoter; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)

IT Gene, microbial
RL: BPR (Biological process); BUU (Biological use, unclassified); BIOL (Biological study); PROC (Process); USES (Uses)
(nuc, Serretia marcescens extracellular nuclease gene; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)

IT Genetic element
RL: BPR (Biological process); BUU (Biological use, unclassified); BIOL (Biological study); PROC (Process); USES (Uses)
(promoter, PL, Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)

IT Ribonucleic acid formation factors
RL: BPR (Biological process); BUU (Biological use, unclassified); BIOL (Biological study); PROC (Process); USES (Uses)
(repressors, temp.-sensitive; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)

IT Gene, microbial
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(ssb, deletion; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)

IT 9003-98-9P, Deoxyribonuclease
RL: BPN (Biosynthetic preparation); BUU (Biological use, unclassified); BIOL (Biological study); PREP (Preparation); USES (Uses)
(Serratia marcescens extracellular enzyme; Escherichia coli thermo-induced controlled suicide using nuclease gene expression regulation by temp.-sensitive promoter)

ALL ANSWERS HAVE BEEN SCANNED

96-049672/05 B04 C06 D16 WACK/ 94.06.10
WACKERNAGEL W *WO 9534643-A1
94.06.10 94EP-108958 *(95.12.21)* C12N 9/22, 15/64, 15/70 // C12N 1/21 (C12R 1:19)

Conditional suicide cells of Escherichia coli contg. plasmid borne nuclease gene - controlled by promoter regulated by a temp. sensitive repressor, undergoes degradation of intracellular DNA at elevated temp. without cell lysis (Eng)

C96-016278 N(JP US) R(AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT SE)

Addnl. Data: WACKERNAGEL W, LORENZ M G, AHRENHOLZ I, JEKEL M

95.06.09 95WO-EP02245

Genetically engineered conditional suicide cells of *E. coli* contain:
(1) a first plasmid (P1) contg. a promoter (Pr) under control of a temp. sensitive repressor (TSR) at ambient, but not elevated temp., and, downstream of Pr, a nuclease gene (NG) that can be expressed from Pr, and
(2) opt. a second plasmid to express a structural gene (SG) other than NG.

Alternatively P1 contains both the Pr-TSR-NG construct and SG under control of another promoter.

BC(4-E8, 4-F10A3E) D(5-H12E, 5-H14A1) .2

Also new is plasmid pAH12.

USE

Genetically engineered cells are potentially useful in e.g. agriculture, waste treatment and prodn. of some raw materials.

ADVANTAGE

Recombinant DNA in the cells can be destroyed by nuclease digestion intracellularly, before it is released from the cells. This means that undesirable survival of engineered organisms or transfer of recombinant DNA to other organisms is prevented.

PREFERRED CELLS

The cells express ≥2 nucleases. NG is partic. from *Serratia narcescens* lacking its leader coding sequence, either the wild type gene or a variant that can kill *E. coli* by causing intracellular DNA degradation.

Pr is partic. the λ PL promoter and TSR is also from λ phage.

WO 9534643-A+

EP 717775

PREFERRED PLASMID

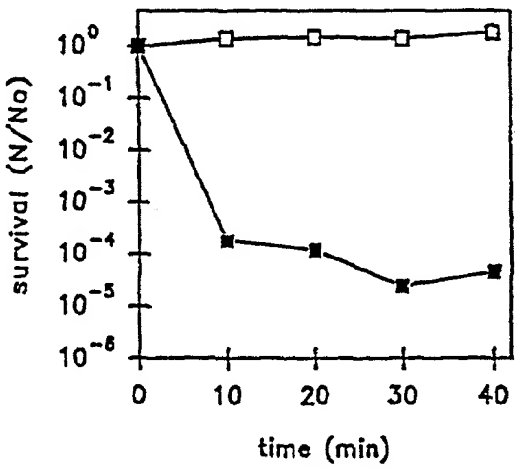
pAH12 is derived from pNuc4 by:
(1)isolating the nuc gene without its leader (as a EagI-BssHI fragment) and insertion into pET81F+, and
(2) isolating, from the recombinant plasmid formed, the nuc gene together with the ribosome binding site and/or T7Phi10 promoter, plus the start codon (as a SspI-BamHI fragment) and inserting this downstream of the PL promoter in plasmid pSF1 (from which the Ssb gene has been detected).

EXAMPLE

Log phase cultures of *E. coli* TGE900 contg. pAH12 were grown at 28 °C, then shifted to 42 °C to inactivate the eI1857 repressor, allowing expression of *S. marcescens* nuclease. Survival of cells was determined by plating (at 28 °C) on ampicillin-contg. agar. The figure (black squares) shows that survival after 30 min. at 42 °C was about 23 cells per million, while *E. coli* TGE900 cells contg. the plasmid pSF1E were unaffected (white squares).

The few cells that survived were themselves sensitive to a second round of heat induction, in most cases about as sensitive as the original cells but in a few cases less so (700 survivors per million after 30 min).

Nuclease-induced destruction of DNA continued for ≥3 hr. after derepression of the truncated nuclease gene and >80% of cellular DNA was converted to acid-soluble prod. without cell lysis. (CW)



(34pp1251DwgNo.2/3)
SR:6.Jnl.Ref EP255755 WO8705932 WO9214819